Cell-Based Assays to Detect the Mechanism of Toxicity

Terry Riss, PhD

Promega

Presentation outline

- Overview of cytotoxicity assays for multi-well plates
  - Live cells
  - Dead cells
  - Apoptotic cells
- Biochemical markers of cell stress
- Multiplexing assays to get more information
- Genetic reporter assays to detect stress response pathways

How do I choose the most appropriate assay?

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First decide what you want to measure

- Number of living cells (viability assay)
- Number of dead cells (cytotoxicity assay)
- Apoptosis vs. necrosis
- Determine events leading up to apoptosis

Understanding how the assays work

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Understanding:

- what the assay is measuring
- how the reagent chemistry works

Will help you predict:

- assay limitations
- potential for artifacts
- compatibility for multiplexing

Methods for measuring cytotoxicity

Promega

Overview of assays to measure viable cells

- MTT / MTS / Resazurin
- Protease marker
- ATP

Assays to detect dead cells

- LDH release
- Protease release
- DNA staining

Assays to measure apoptotic cells

- Caspase activity

Multiplex assays to measure early markers of cytotoxicity

- Viable, dead & apoptotic cells
- Extrinsic & intrinsic apoptosis pathways
- Mitochondrial toxicity
- Oxidative stress
- Proteasome activity

Luciferase reporters of cell stress pathways leading to cytotoxicity

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Luciferase reporters of cell stress pathways leading to cytotoxicity

Common metabolic indicators of cell viability

- **Indicator dyes (MTT / MTS / Resazurin)**: NADH in metabolically active viable cells can reduce tetrazolium compounds into brightly colored formazan products or reduce resazurin into fluorescent resorufin.
- **Protease marker**: Aminopeptidase activity present only in viable cells can be measured using a cell permeable fluorogenic substrate.
- **ATP** is present in all cells and has been established as a valid marker of cell viability. ATP is measured using a beetle luciferase reaction to generate light.

Metabolic indicators of cell viability

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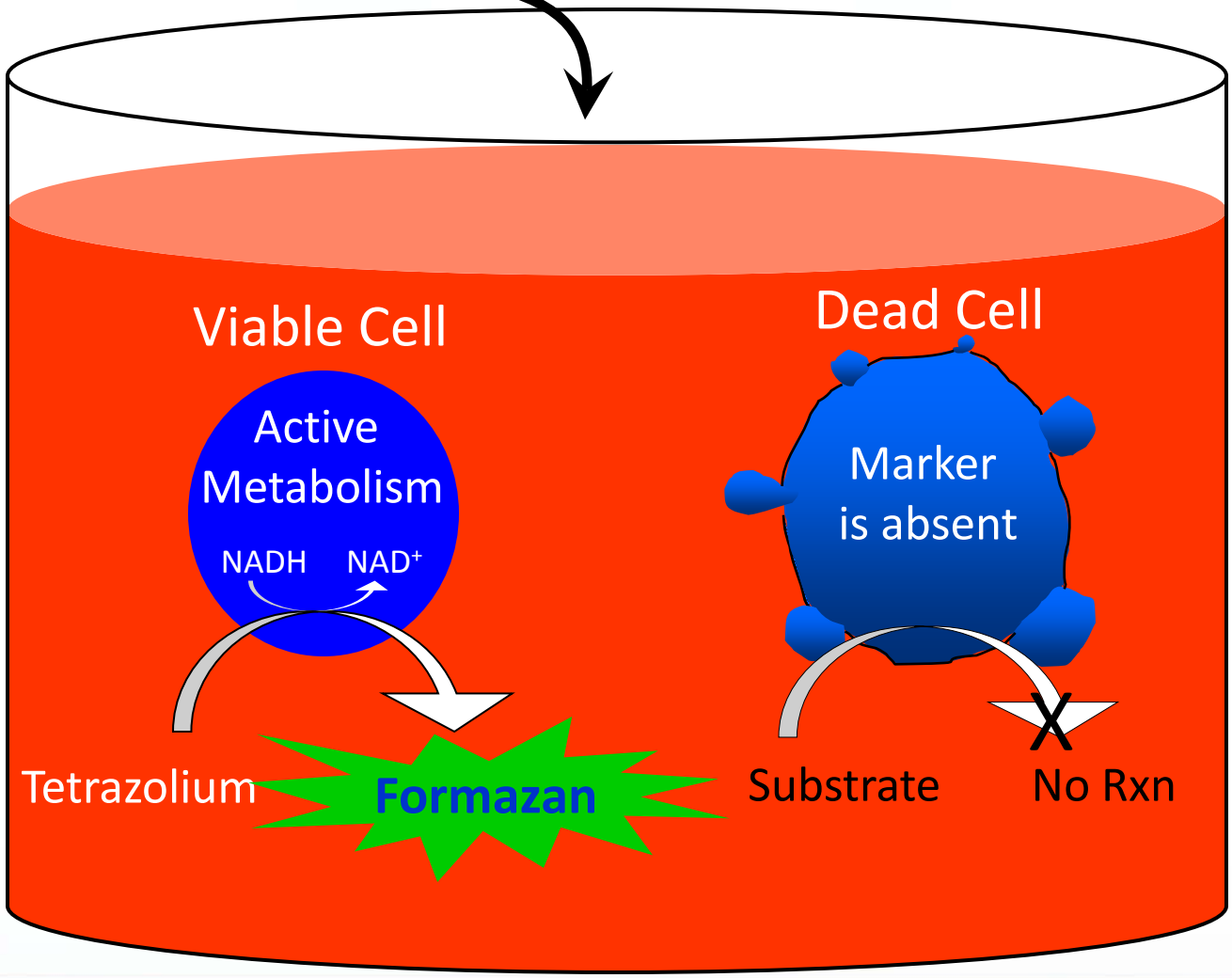
Tetrazolium Reagent

Tetrazolium Reagents

- **MTT**
- **MTS**
- **XTT**
- **WST-1**



Metabolic indicators of cell viability

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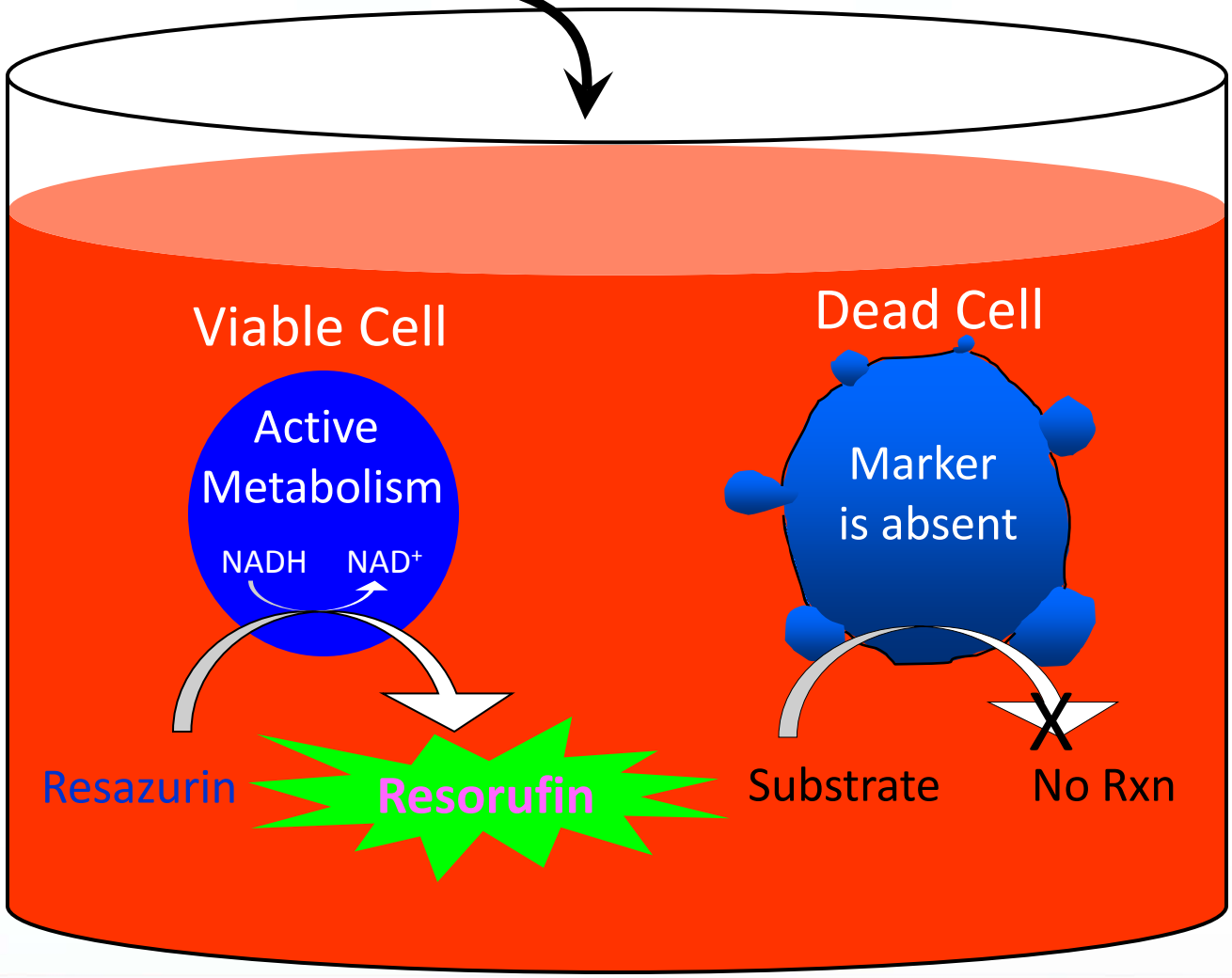
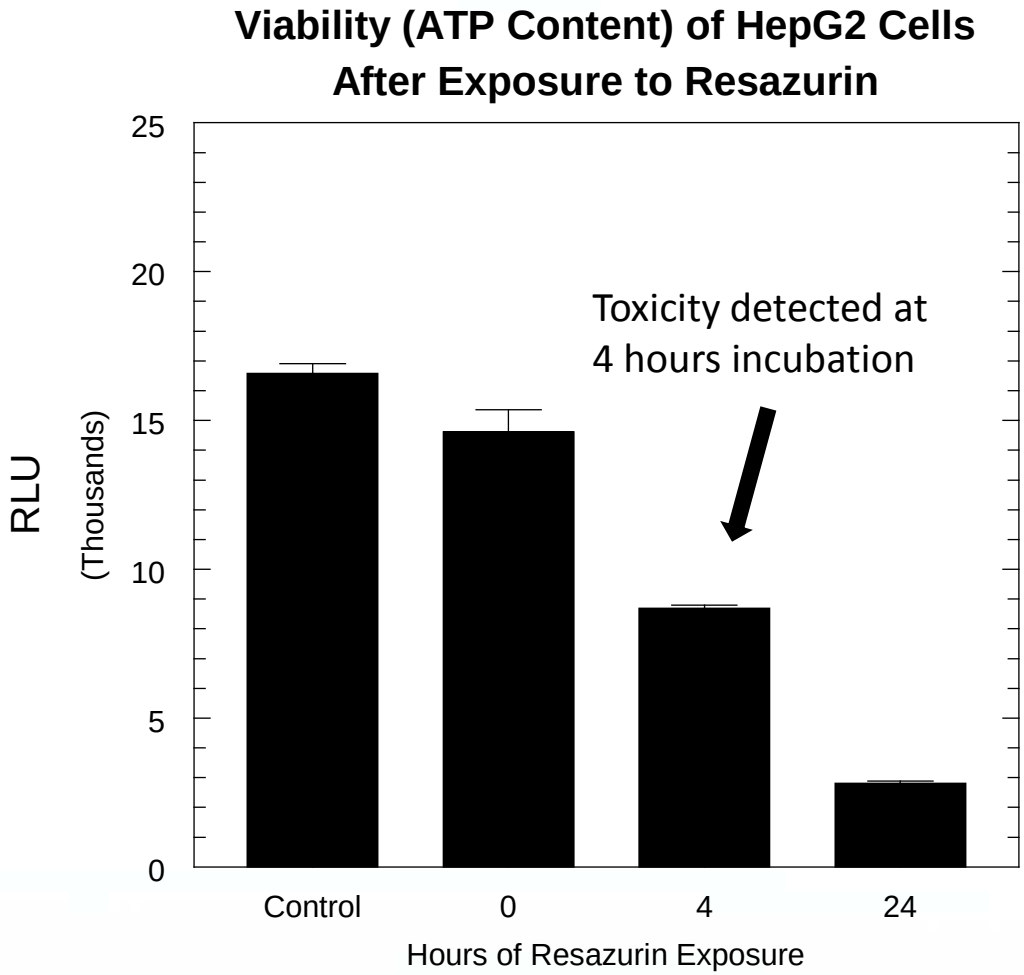
Resazurin

Redox Indicators

- **Resazurin**



Prolonged exposure of cells to tetrazolium or resazurin reagents results in toxicity



Metabolic indicators of cell viability

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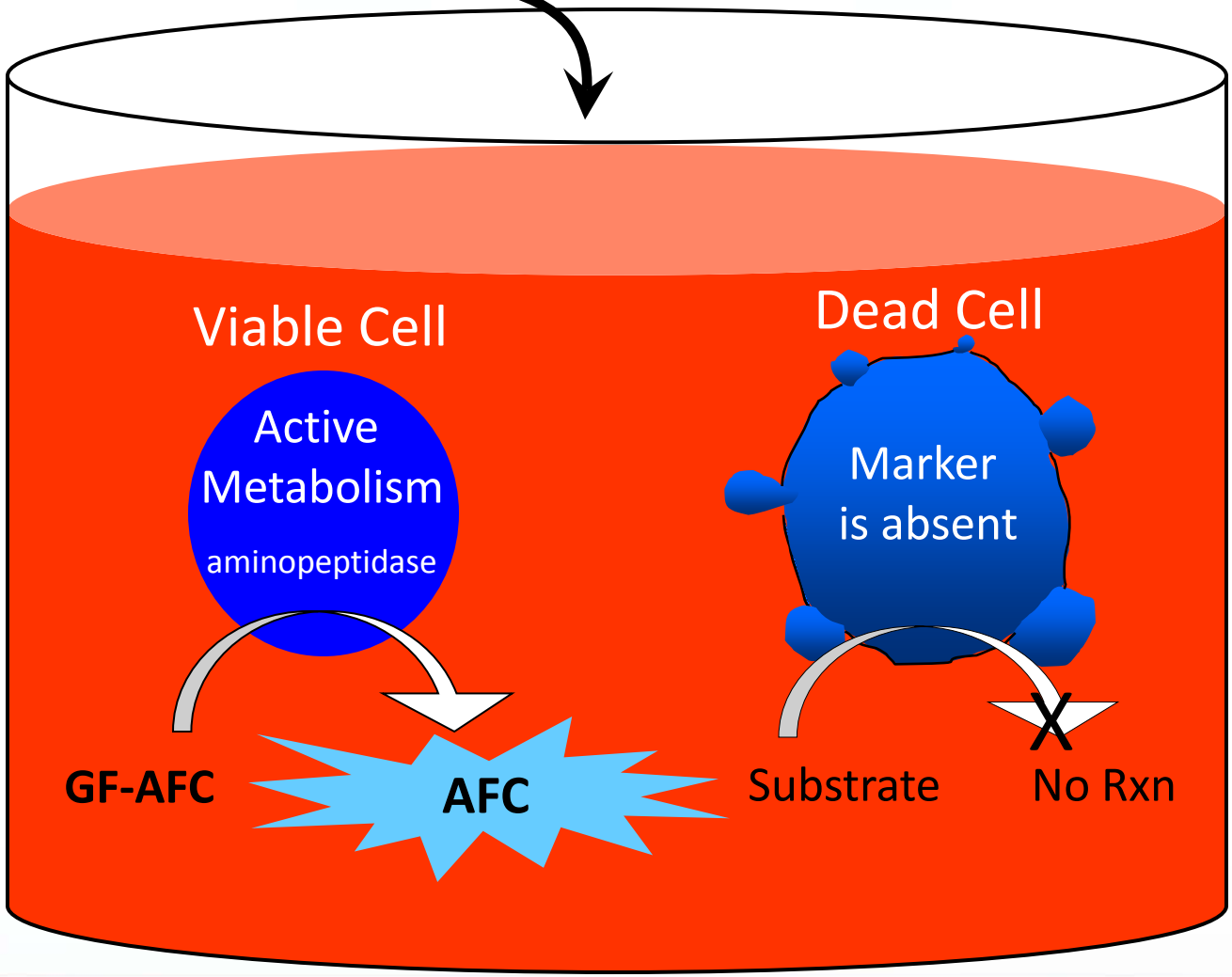
GF-AFC

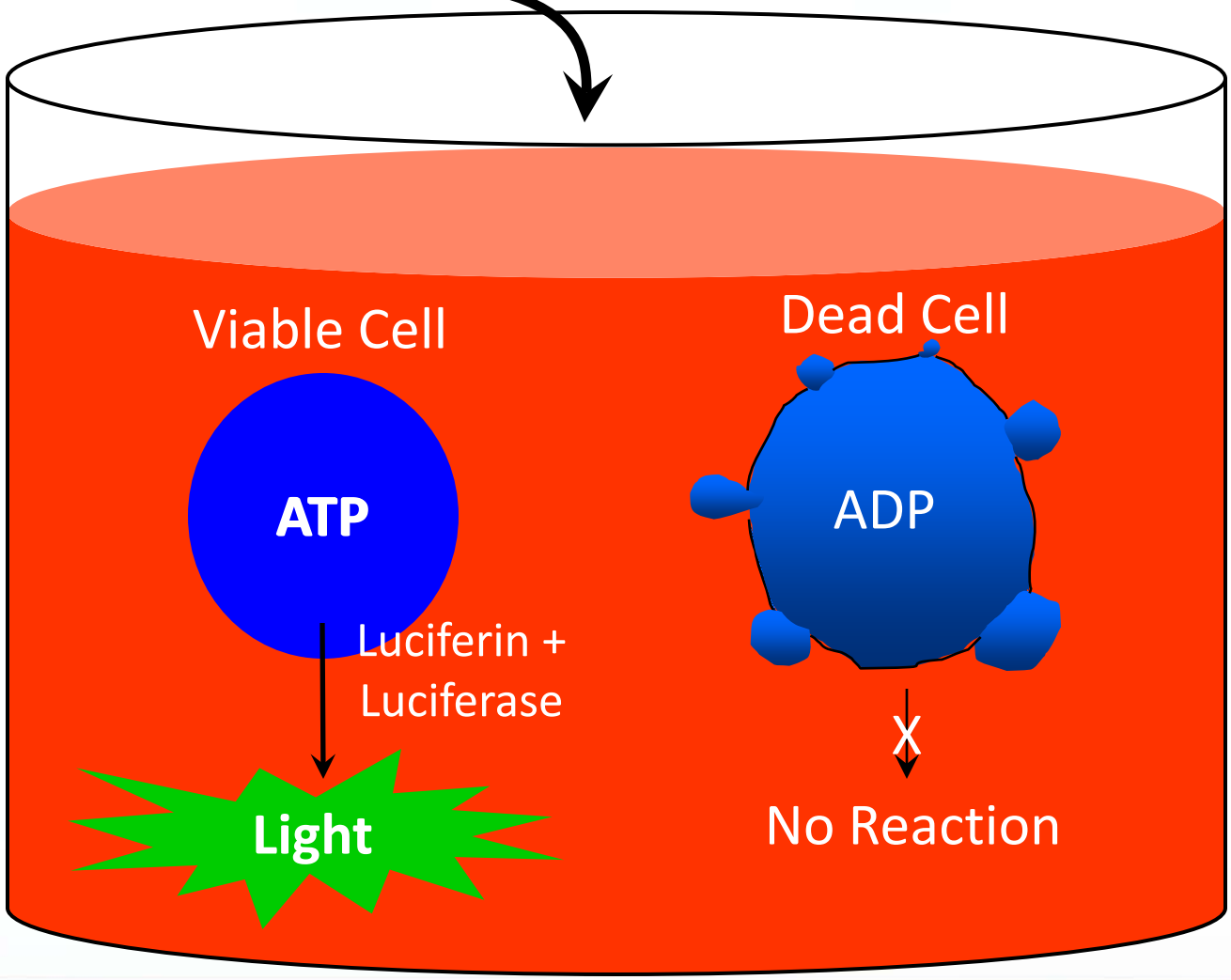
Enzyme Substrates
- **Protease Substrates**



ATP assay for cell viability

CellTiter-Glo® Reagent

- Lysis Solution
- ATPase Inhibitors
- Luciferin
- UltraGlo Luciferase



Advantages & disadvantages of viability assays

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Assay	Advantages	Disadvantages
MTT / MTS	Widely used Inexpensive	1-4 hour incubation Interference by reducing compounds 2 step protocol (MTT) Toxic to cells Limited sensitivity
Resazurin	Inexpensive Fluorescent readout Good sensitivity	1-4 hour incubation Interference by reducing compounds Toxic in some cases* Fluorescence interference
Protease	30 min protocol Better sensitivity than resazurin Cells remain viable Good choice for multiplexing	Fluorescence interference
ATP	10 min protocol Best sensitivity No interference by fluorescent compounds Stops reaction immediately	Lytic protocol dictates sequence for multiplexing

Methods for measuring cytotoxicity

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Overview of assays to measure viable cells

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- ATP

Assays to detect dead cells

- **LDH release**
- **Protease release**
- **DNA staining**

Assays to measure apoptotic cells

- Caspase activity

Multiplex assays to measure early markers of cytotoxicity

- Viable, dead & apoptotic cells
- Extrinsic & intrinsic apoptosis pathways
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- Oxidative stress
- Proteasome activity

Luciferase reporters of cell stress pathways leading to cytotoxicity

Assays to detect dead cells

The functional definition of cell viability is based on whether the outer membrane is intact

Membrane integrity and thus dead cells can be detected by:

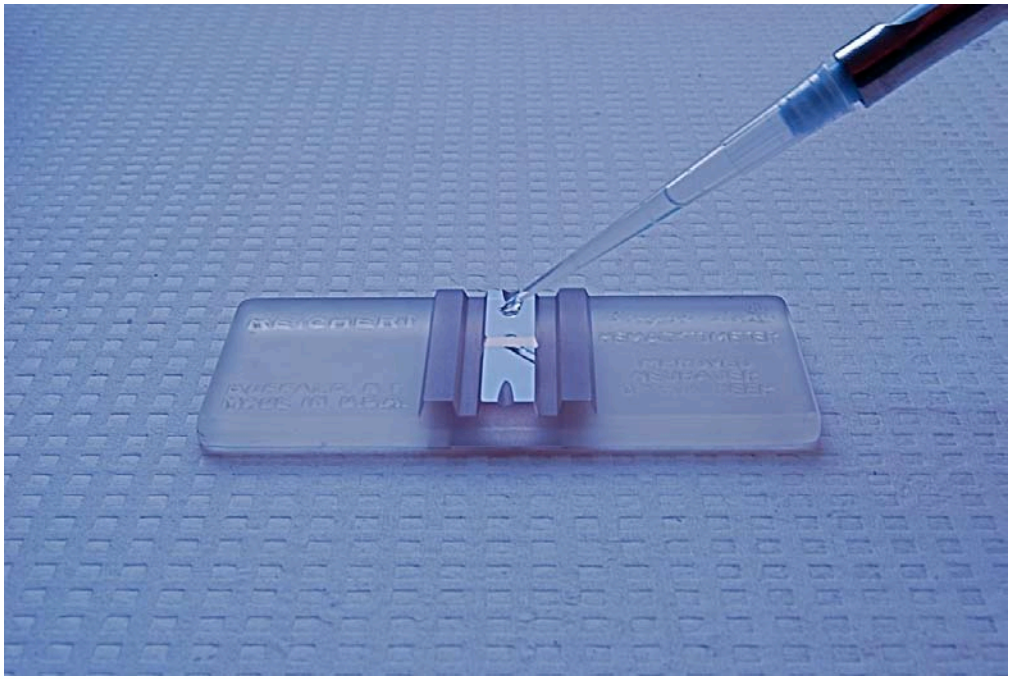
- Measuring activity of marker enzymes that leak out of dead cells into the culture medium
- Observing staining of cytoplasmic or nuclear content by vital dyes that can only enter dead cells

Loss of membrane integrity defines a "dead" cell

Trypan blue staining to determine percent viability may be the most common "assay" used in cell culture labs.

Trypan blue is not permeable to live cells.

Dead cells that have lost membrane integrity take up dye and stain blue.



LDH release assay to detect dead cells

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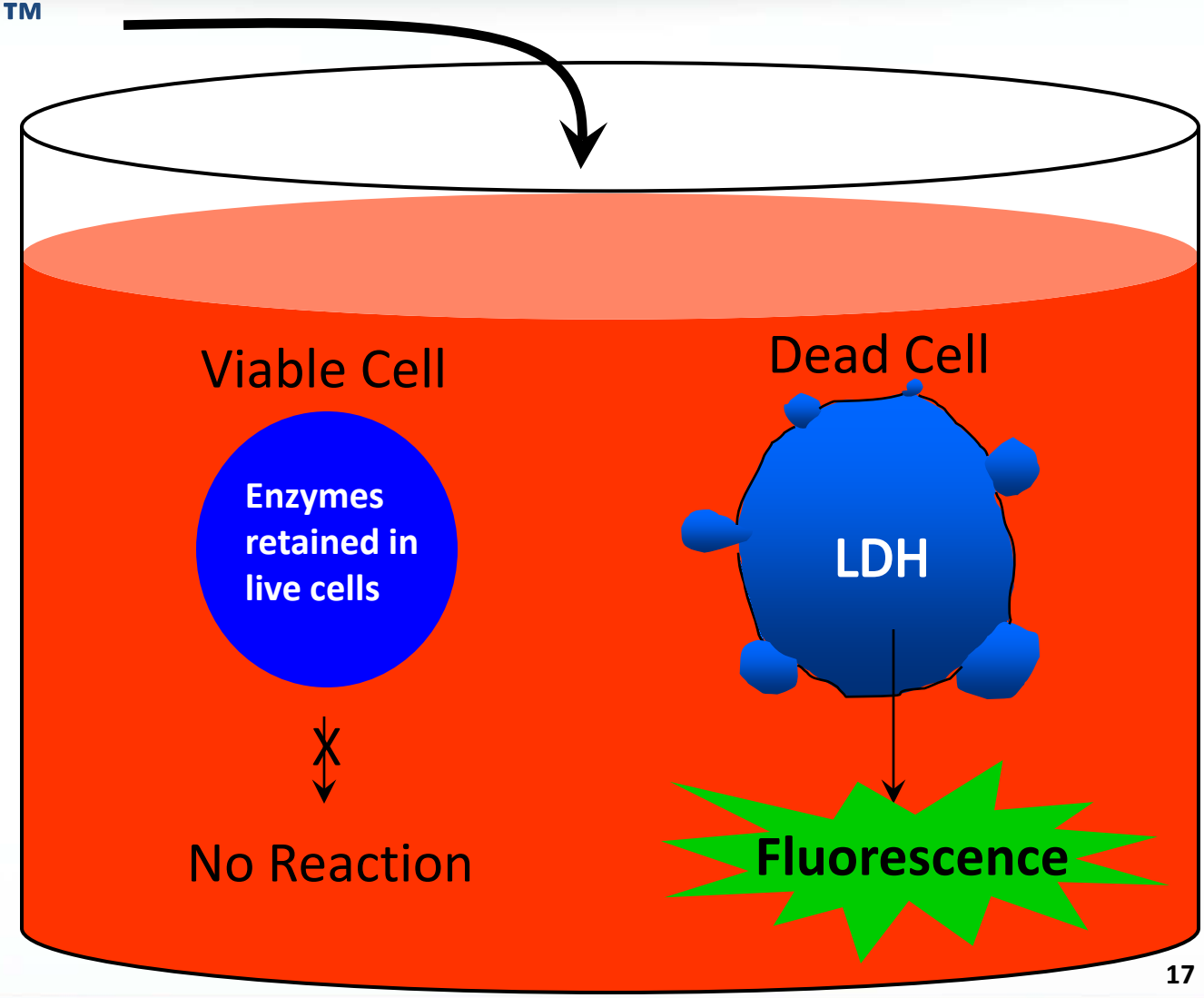
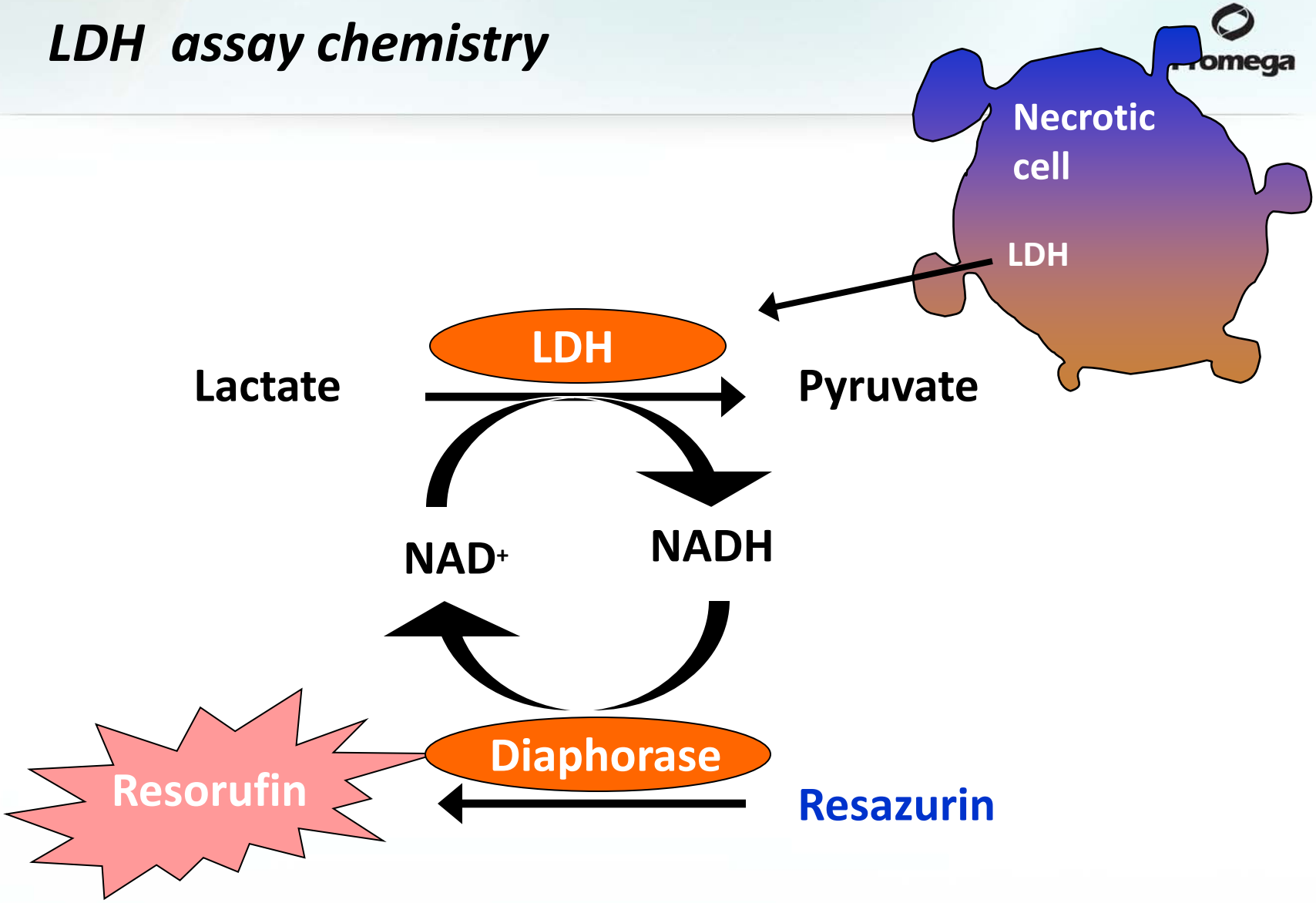
CytoTox-ONE™ Reagent*

- Lactate
- NAD^+
- Diaphorase
- Resazurin

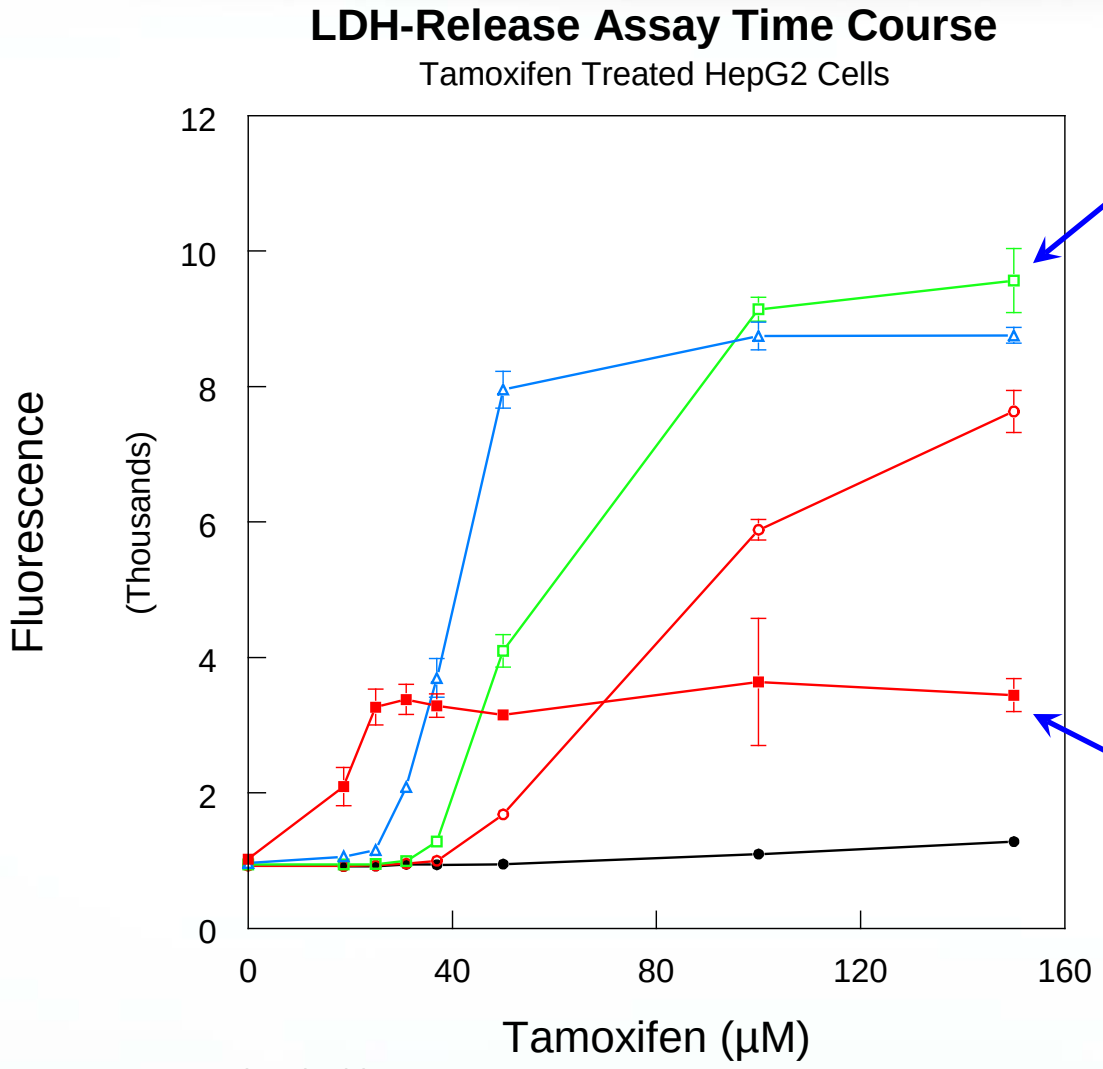


*US Patent 6,982,152 B2

LDH assay chemistry



Stability of released enzyme activity in culture medium becomes a limitation



Cells are all dead at 2 hr treatment with 150µM Tamox

LDH activity in medium decreases after 24 hours

See: Assay & Drug Devel Tech 2(1): 51, 2004

Protease release assay to detect dead cells

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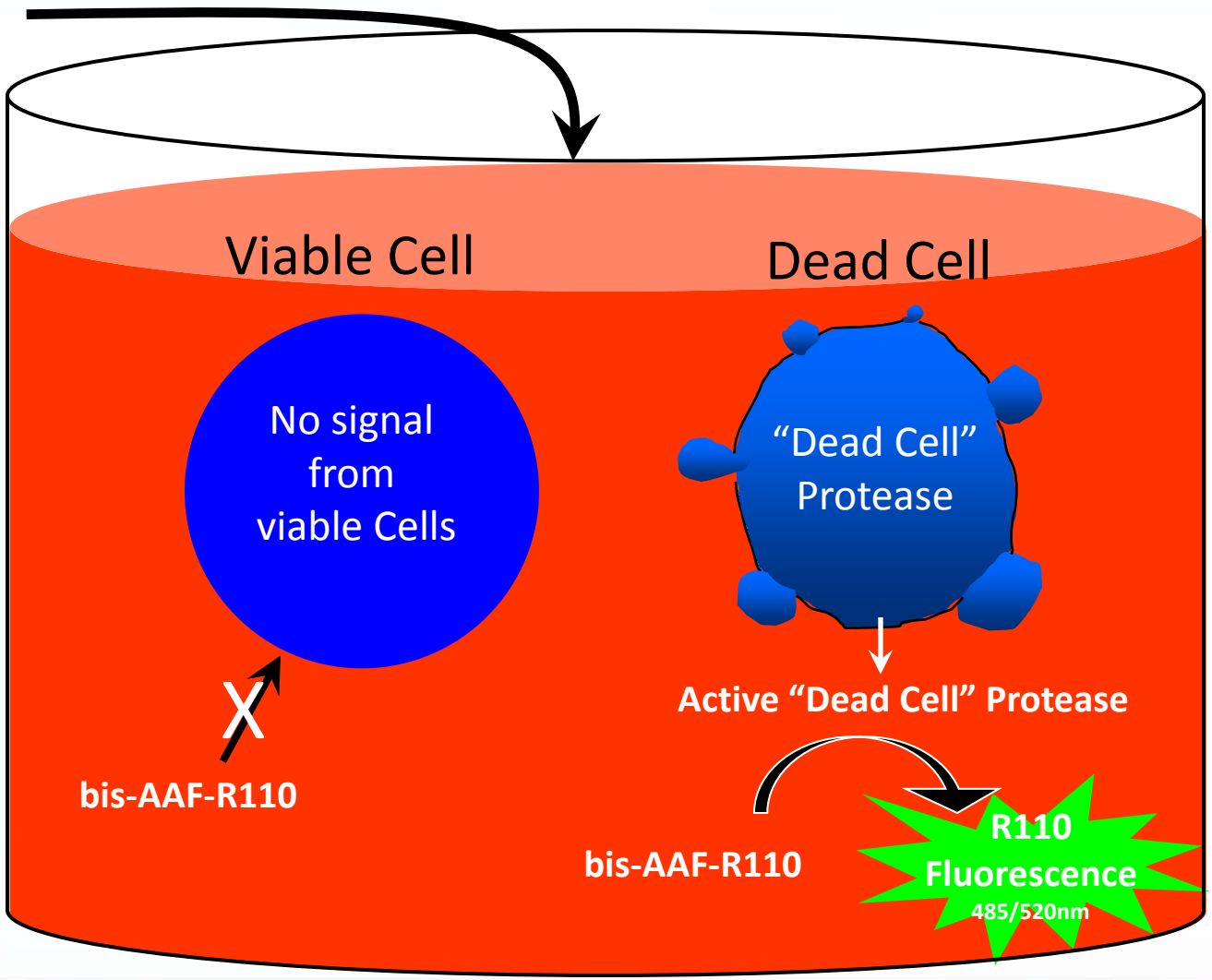
- Impermeable substrate can not enter viable cells
- "Dead" Cell protease remains active after cell death
- The only signal is from "Dead" Cells



DNA dye staining to detect dead cells

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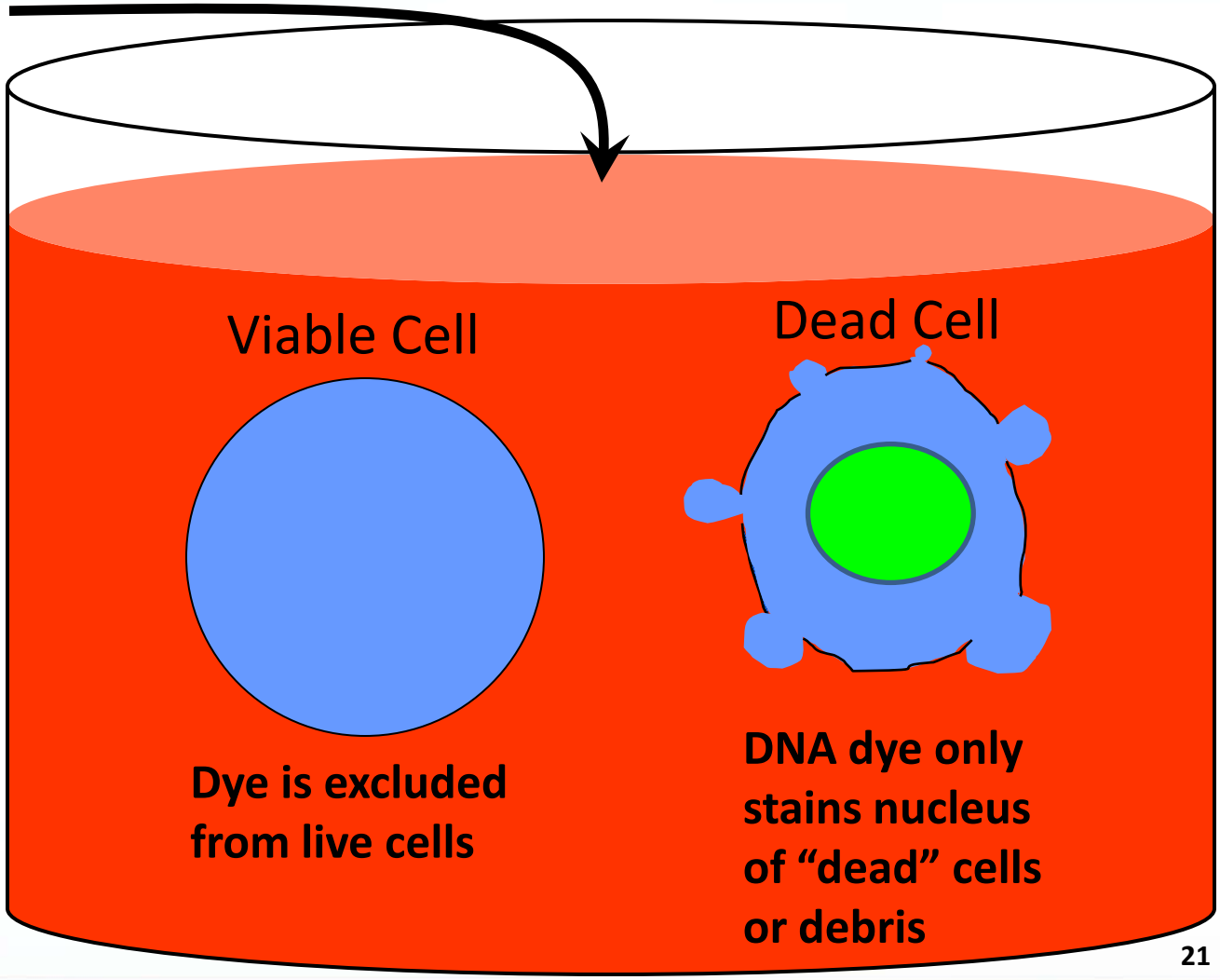
Non-permeable DNA dye

Staining of dead cells results in a fluorescent signal.

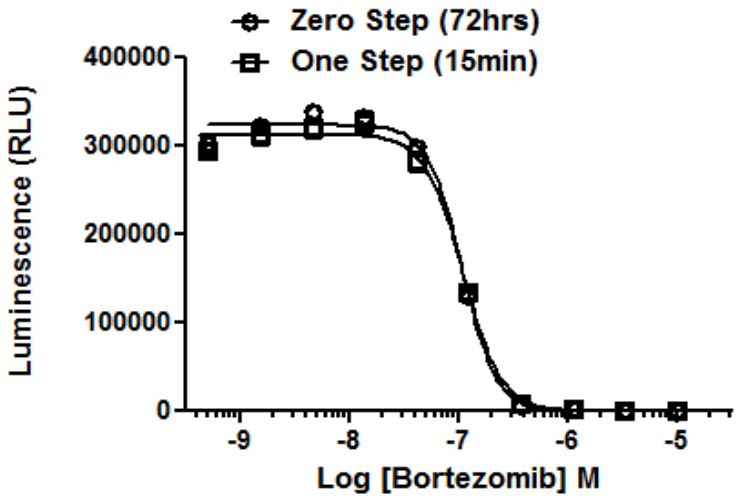


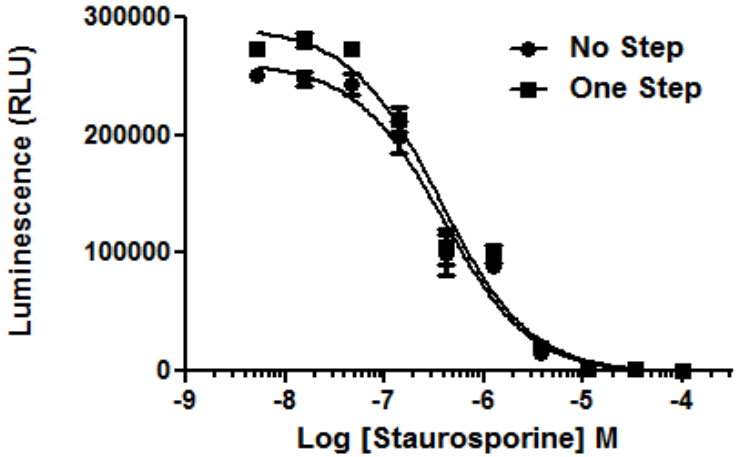
Advantage of DNA staining = signal stability

- DNA staining of dead cells produces a fluorescent signal that lasts much longer than the signal from enzyme release assays.
- When marker enzymes are released from dead cells, enzymatic activity diminishes over time in culture medium (~9hr half-life)
- Promega's new DNA staining dye overcomes the major disadvantage of enzyme release assays.
- CellTox Green product will be commercially available soon

Promega's DNA dye is not toxic to cells

Incubation of DNA dye with cells for 72 hours has no effect on viability measured using the CellTiter-Glo ATP Assay





DNA dye does not effect IC_{50} of model compounds in 72hr co-incubations.

Multiplexing DNA staining and ATP assays

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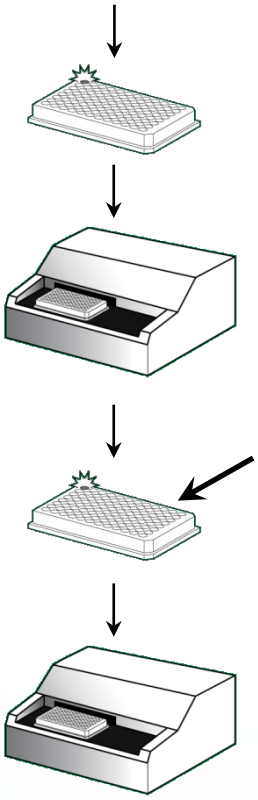
Add DNA dye
when seeding cells

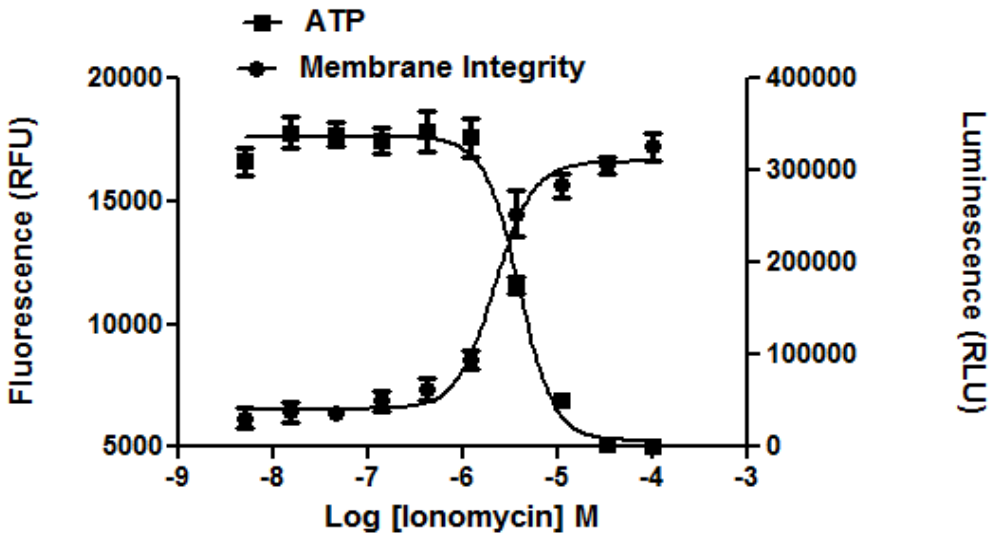


Incubate
72hr

Record
fluorescence
from dead cells

Add CellTiter-Glo® Reagent

Record
Luminescence
from live cells



	Membrane Integrity	ATP
IC50	2.177e-006	3.950e-006

Advantages & disadvantages of assays to detect dead cells

Assay	Advantages	Disadvantages
LDH release	Widely used and accepted Absorbance or fluorescent options	Limited sensitivity Limited half-life of LDH in medium
Protease release	Designed for multiplexing More sensitive than LDH Fluorescent reagent is simpler than formulation for LDH assay Fluorescent or luminescent options	Limited half-life of protease marker Fluorescence interference (fluorescent format only)
DNA Staining	Non-toxic / real time assay Staining persists for 72 hours Good choice for multiplexing	Fluorescence interference Less sensitive than amplified protease release assay

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Promega

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Multiplex assays to measure early markers of cytotoxicity

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Luciferase reporters of cell stress pathways leading to cytotoxicity

Assays to detect apoptotic cells

- Apoptosis is a form of programmed cell death that can be triggered by a variety of signal transduction pathways
- Caspase family members are involved in upstream signal transduction events as well as the systematic dismantling of structural and functional components of the cell.
- Caspase-3 is the predominant “executioner” protease in apoptotic cells and is considered a “universal” marker of apoptosis
- Caspase-3 activity can be easily measured using homogeneous fluorescent or luminescent multi-well plate assays

AMC, R110 and aminoluciferin substrates for measuring caspase activity

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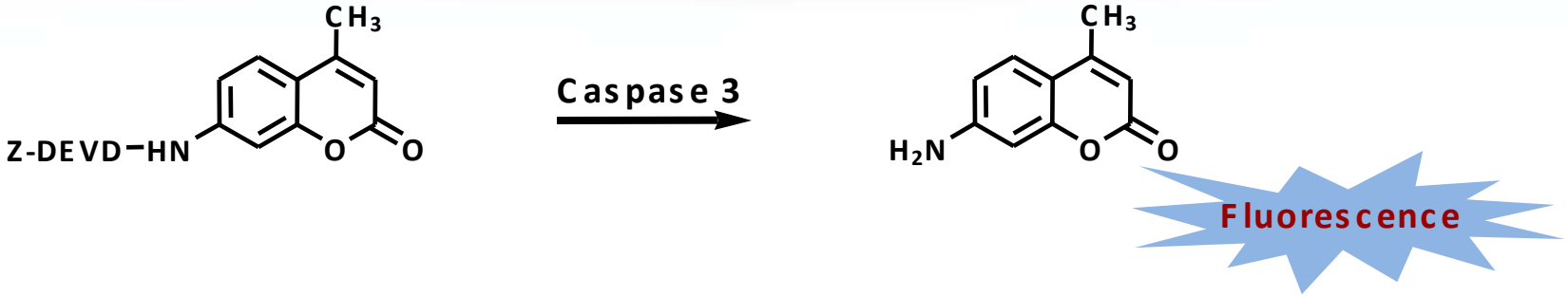
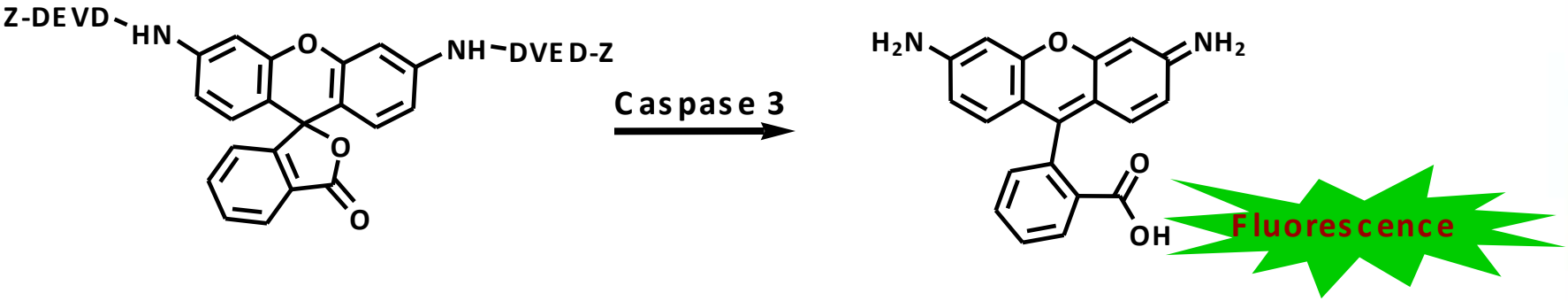
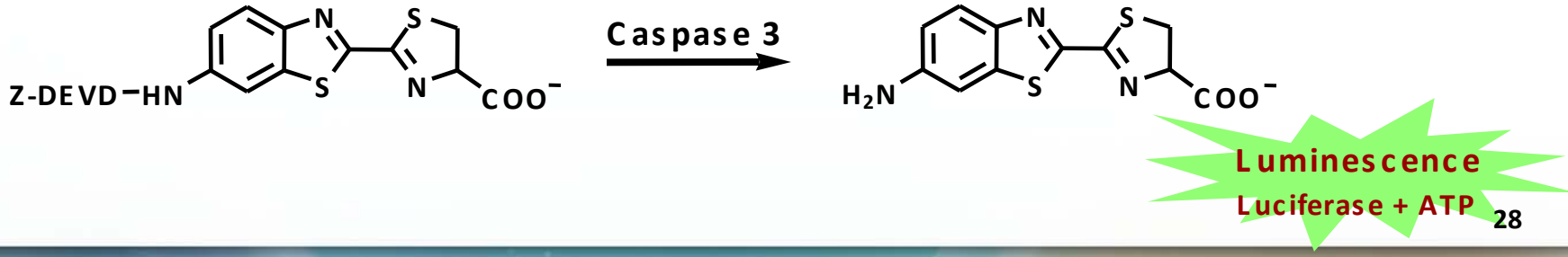
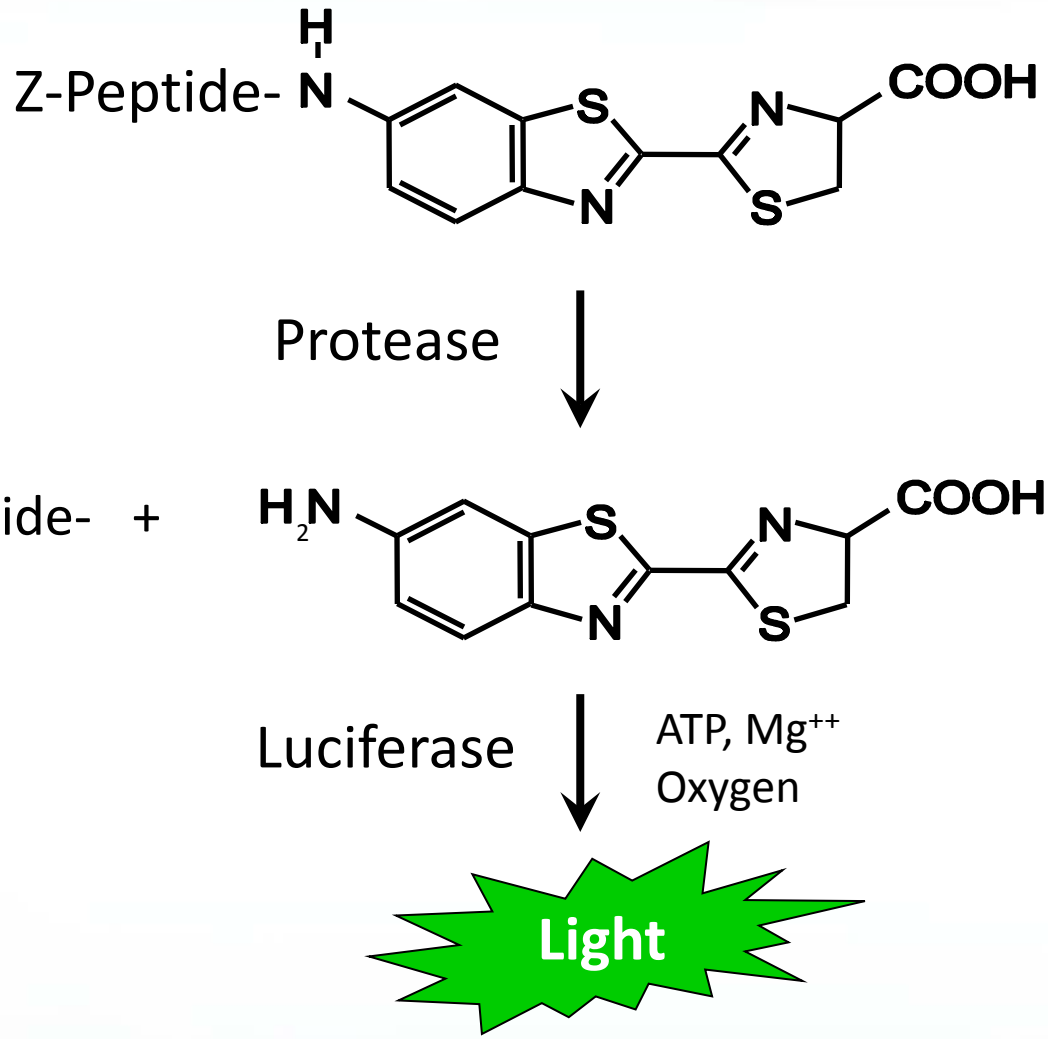






Bioluminescent protease assay chemistry



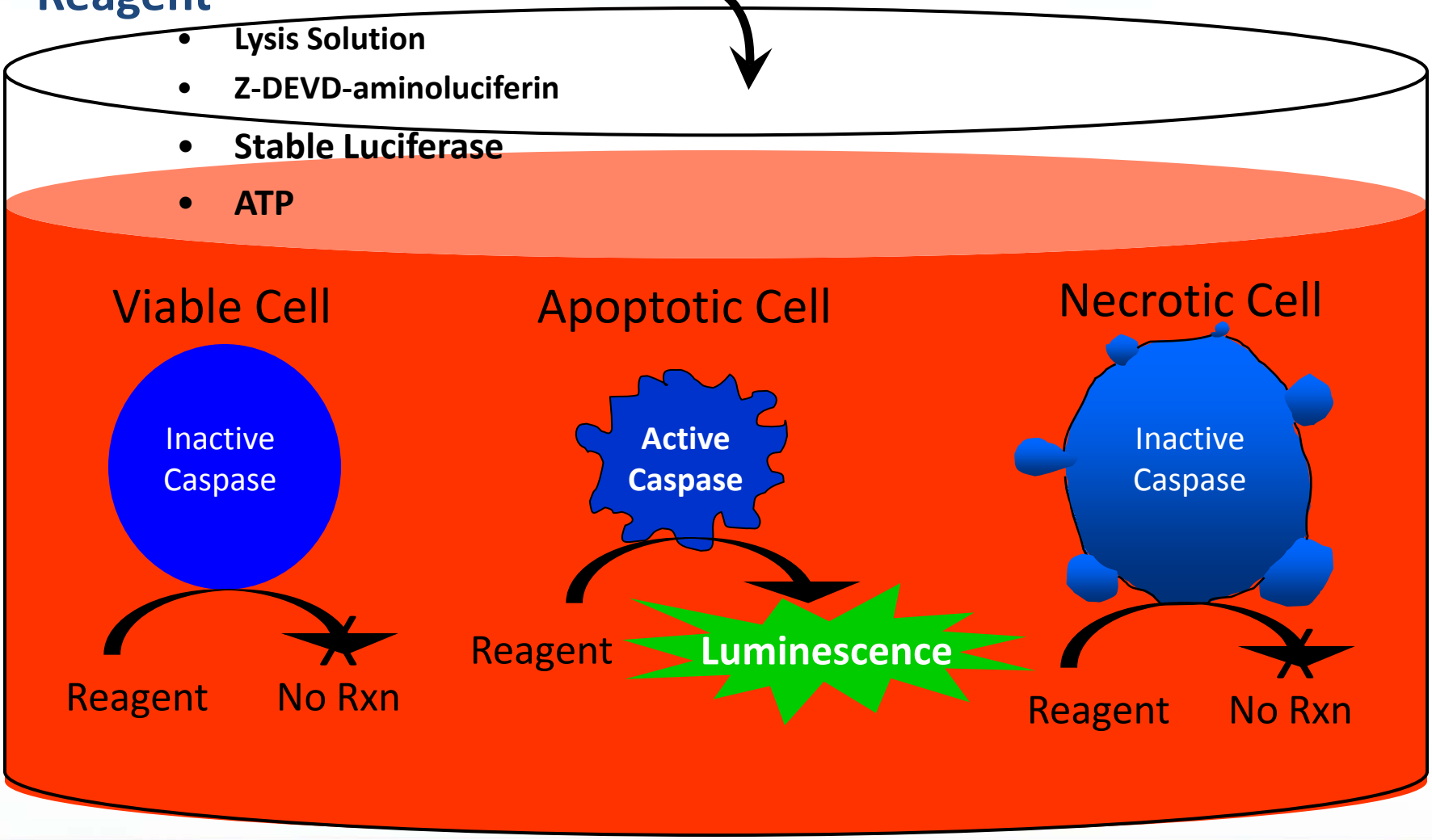
Peptide-aminoluciferin is not a substrate for luciferase

Luminescent caspase assay

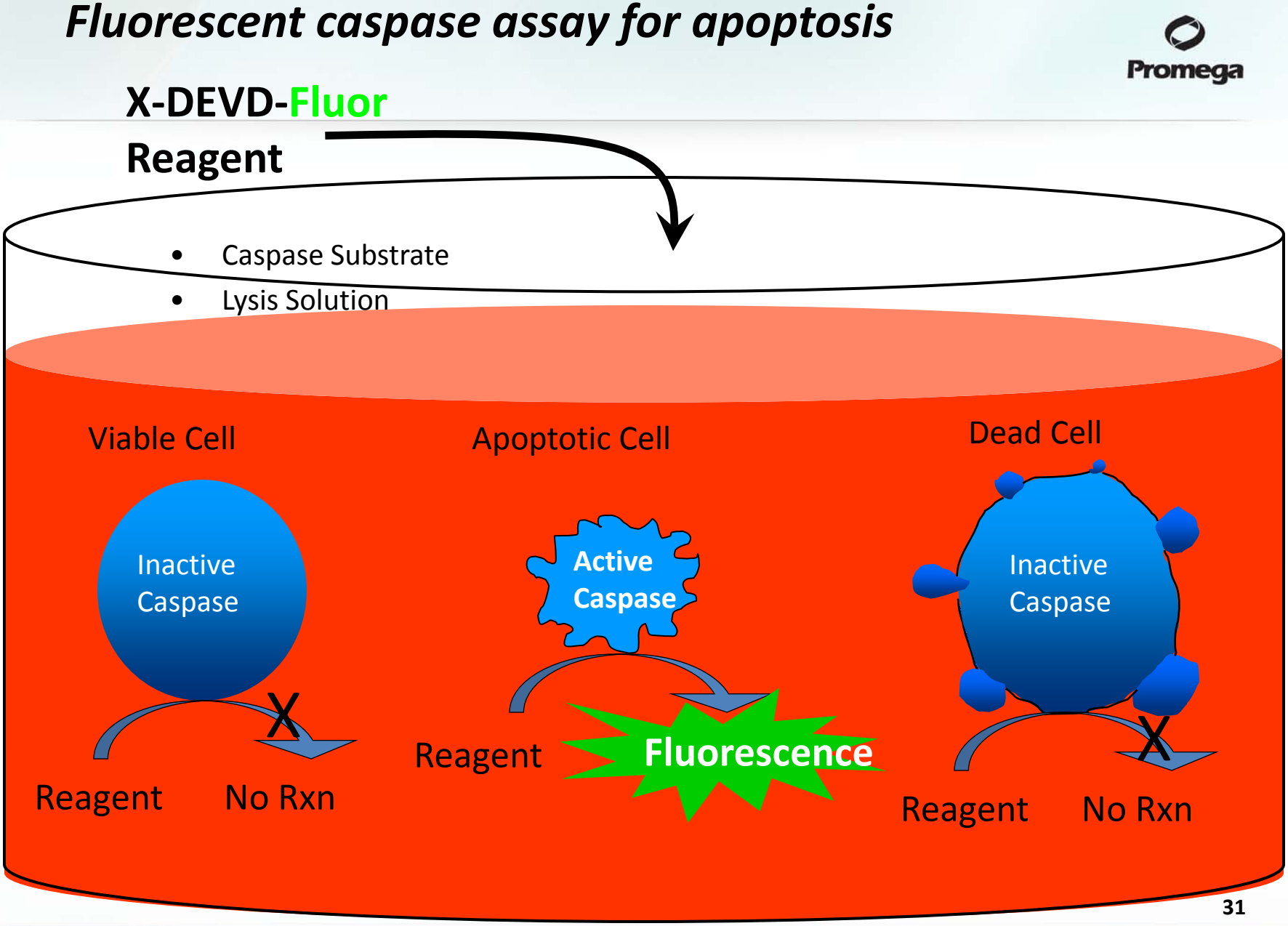
Promega

Caspase-Glo® Reagent

- Lysis Solution
- Z-DEVD-aminoluciferin
- Stable Luciferase
- ATP



Fluorescent caspase assay for apoptosis



Comparison of luminescent and fluorescent protease assay sensitivity

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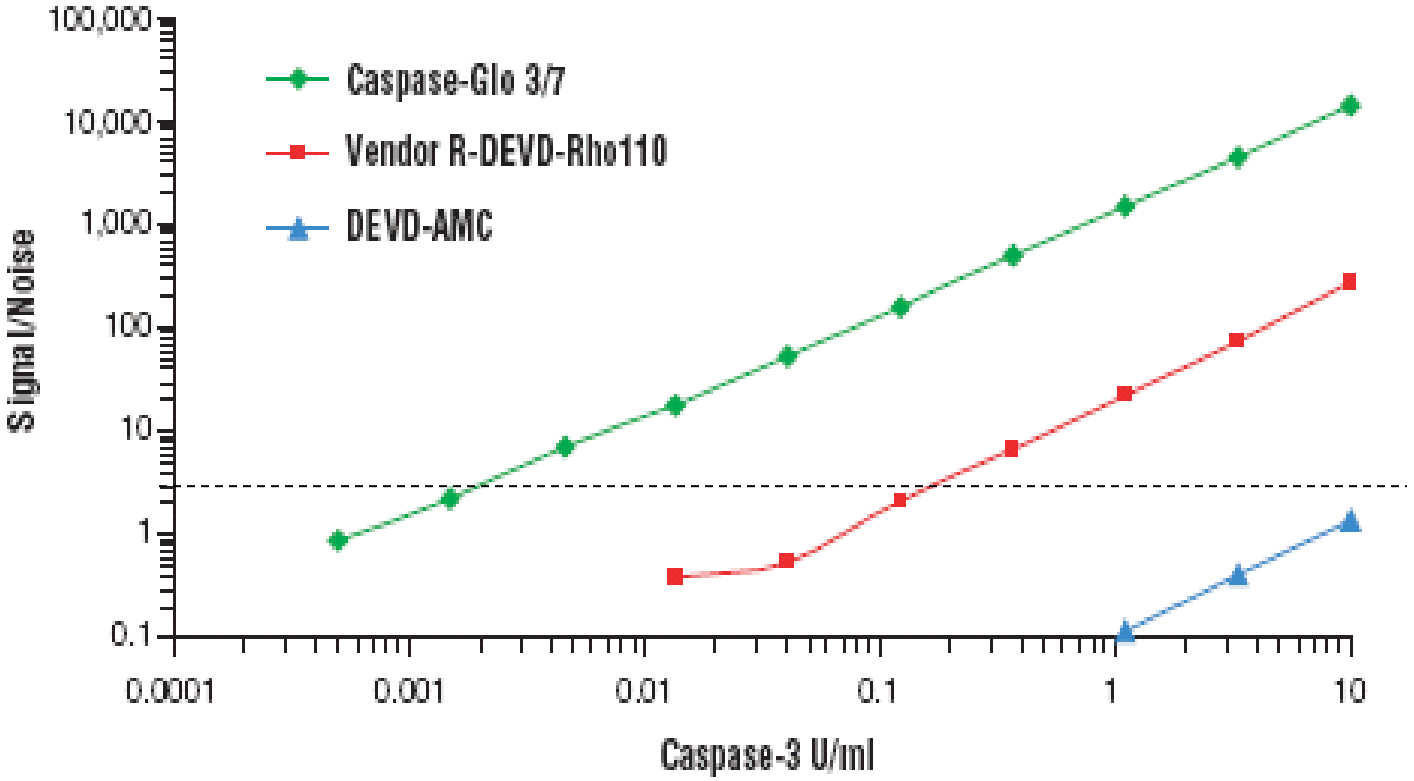
Same “DEVD” protease substrate, but three different ”R” groups



Luminescence is 50-1000-fold more sensitive than fluorescent assays
Low background & long linear range of response

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Promega

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- **Oxidative stress**
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Luciferase reporters of cell stress pathways leading to cytotoxicity

Multiplexing is…

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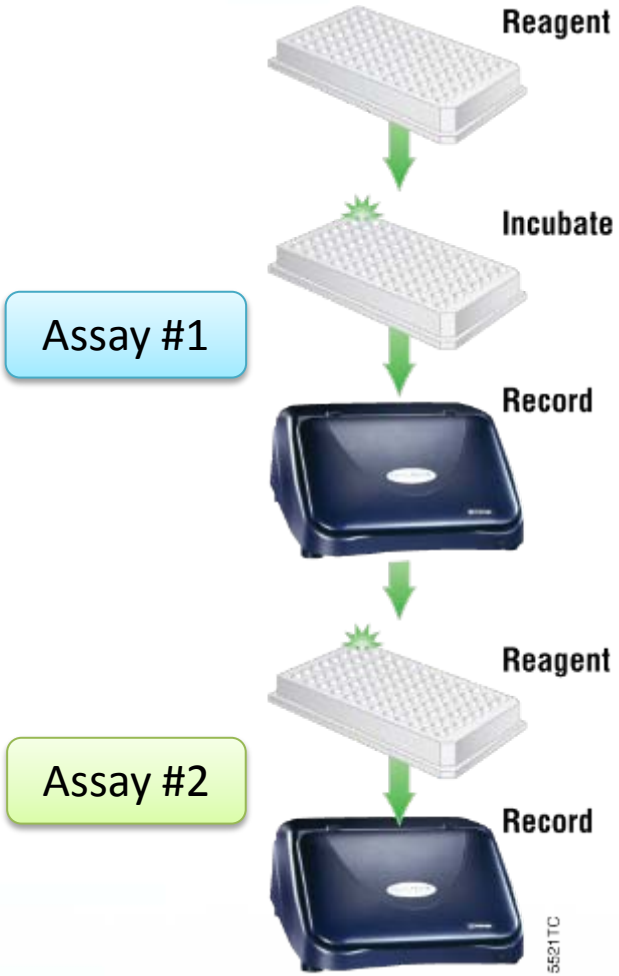
Gathering more than one set of data from the same sample

Multiplexing requirements:

- Assays must be biologically & chemically compatible
- Signals must be spectrally distinct



Why do multiplexing?

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Multiplexing gives a more complete picture of what's happening in the sample

- Reduces cell culture effort to do more than one assay on the same sample of cells
- Can confirm results with two independent methods
- Can normalize assay signal to viable (or total) cell number
  - Correct for cell dispensing errors
  - Differential growth of cells & edge effects

Multiplex detection of viable, dead and apoptotic cells to determine mechanism of cell death

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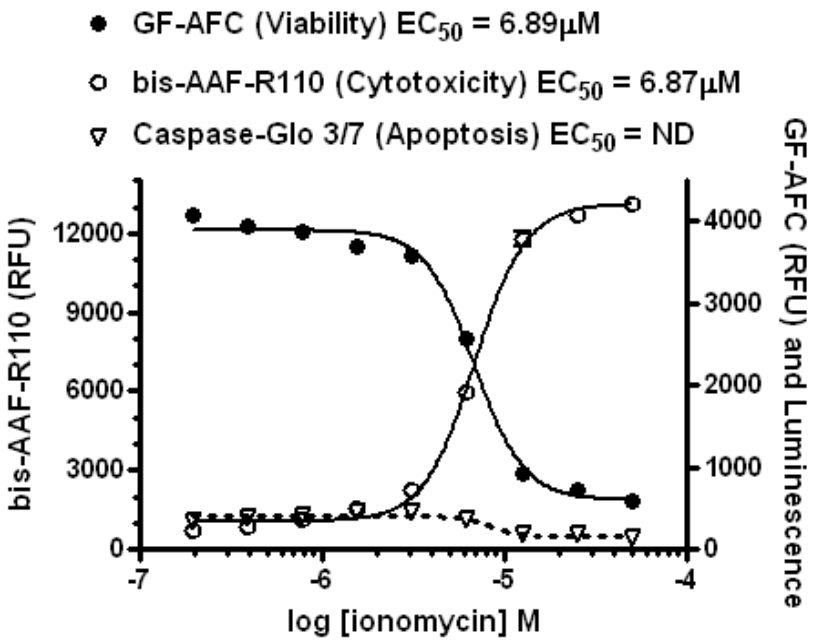
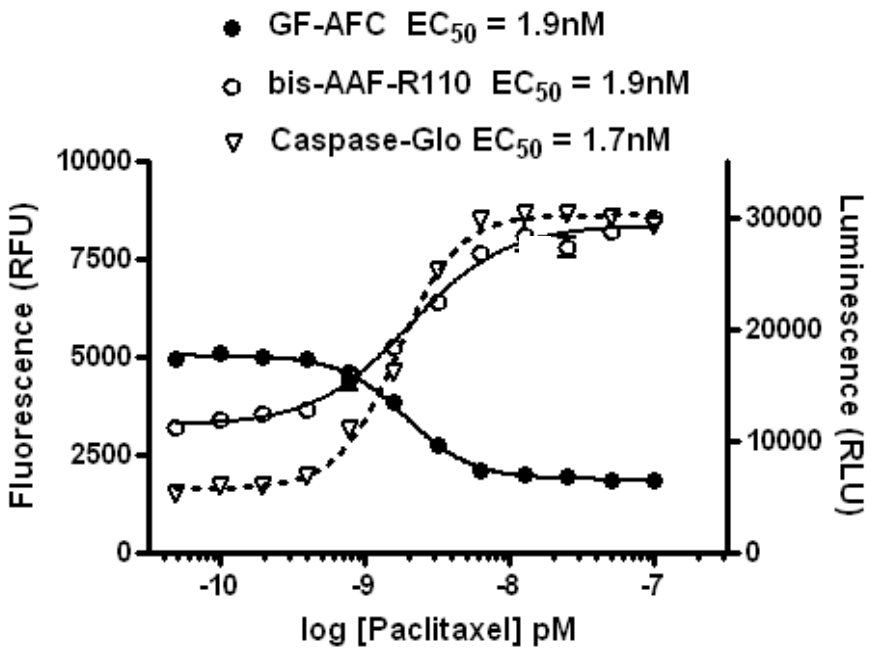
Primary necrosis
(No caspase activity)



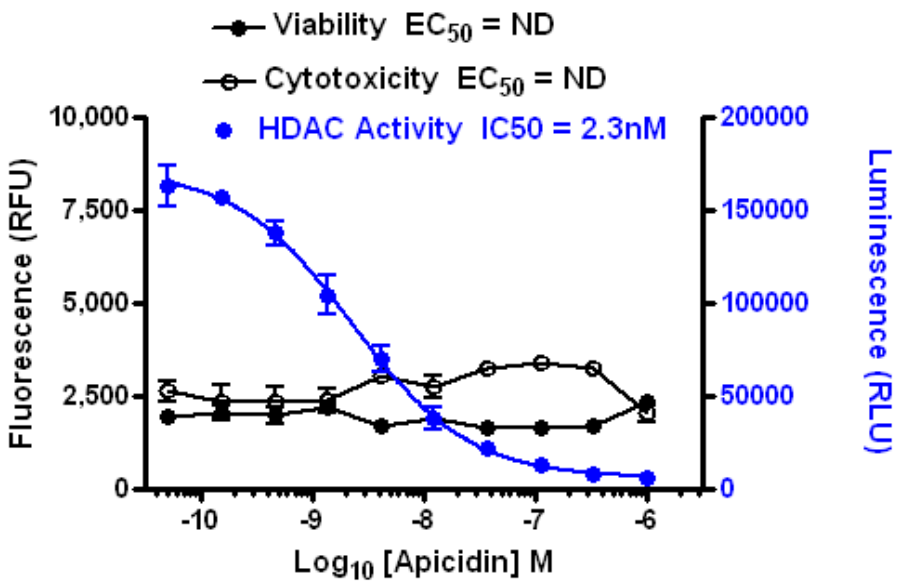
Apoptosis
(Secondary necrosis)



Detecting off-target toxicity by multiplexing MultiTox-Fluor followed by HDAC-Glo

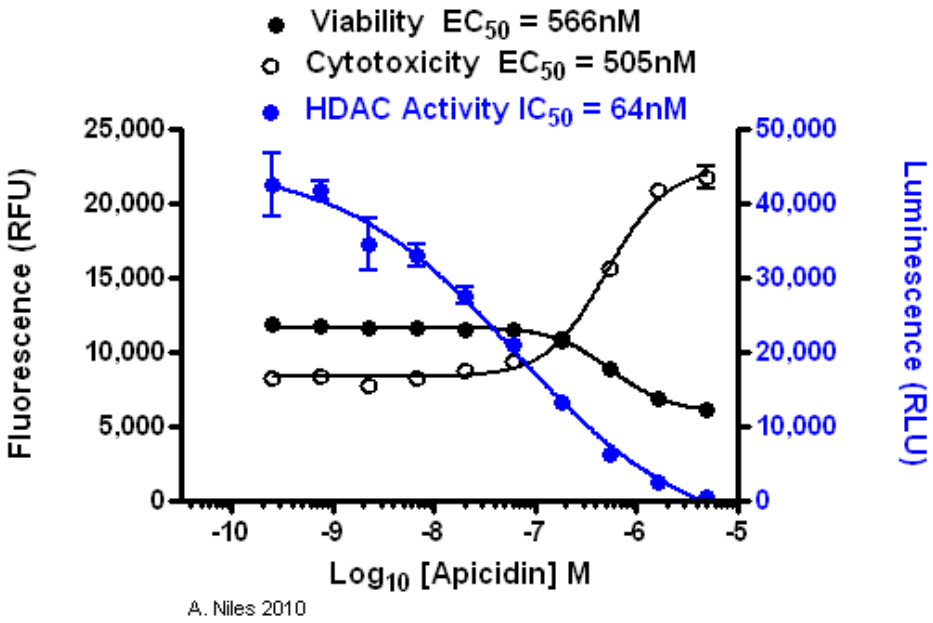
Promega

Cardiomyocytes (Primary)



C. Zimprich 2010

U937 (Transformed Cancer Cells)



A. Niles 2010

[24hr compound exposure]

Intrinsic and extrinsic apoptotic pathways terminating with activation of Caspase-3 → Apoptosis

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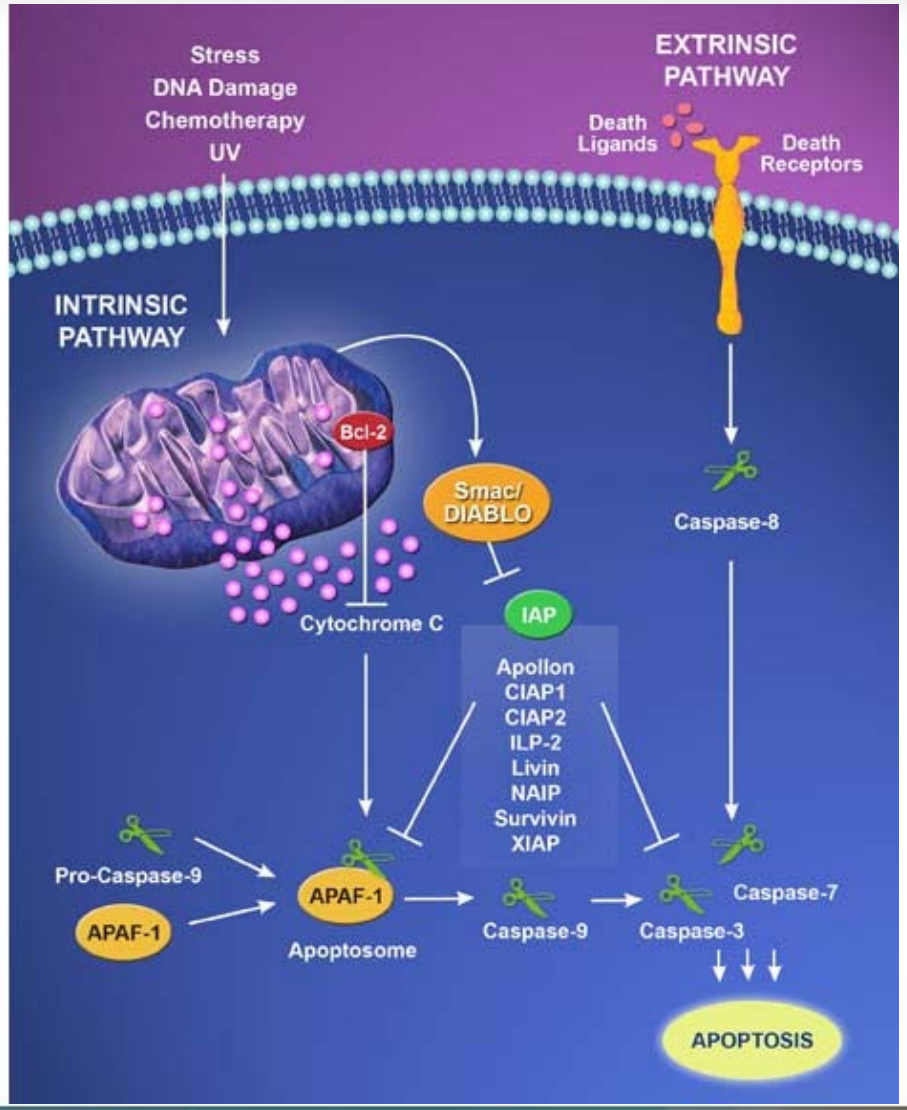

http://www.imgenex.com/emarketing/103006_lymphoma/apoptosis-pathway.jpg

Multiplex assay of signaling and executioner caspases

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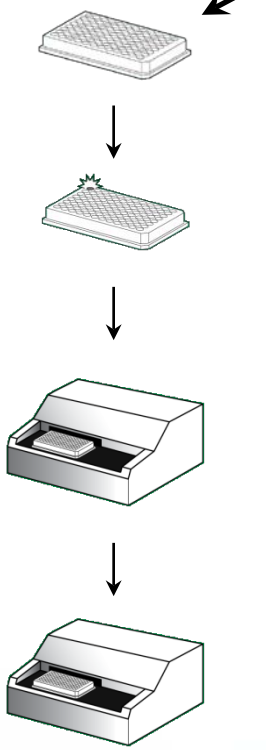
Combining fluorogenic and luminogenic protease substrates together

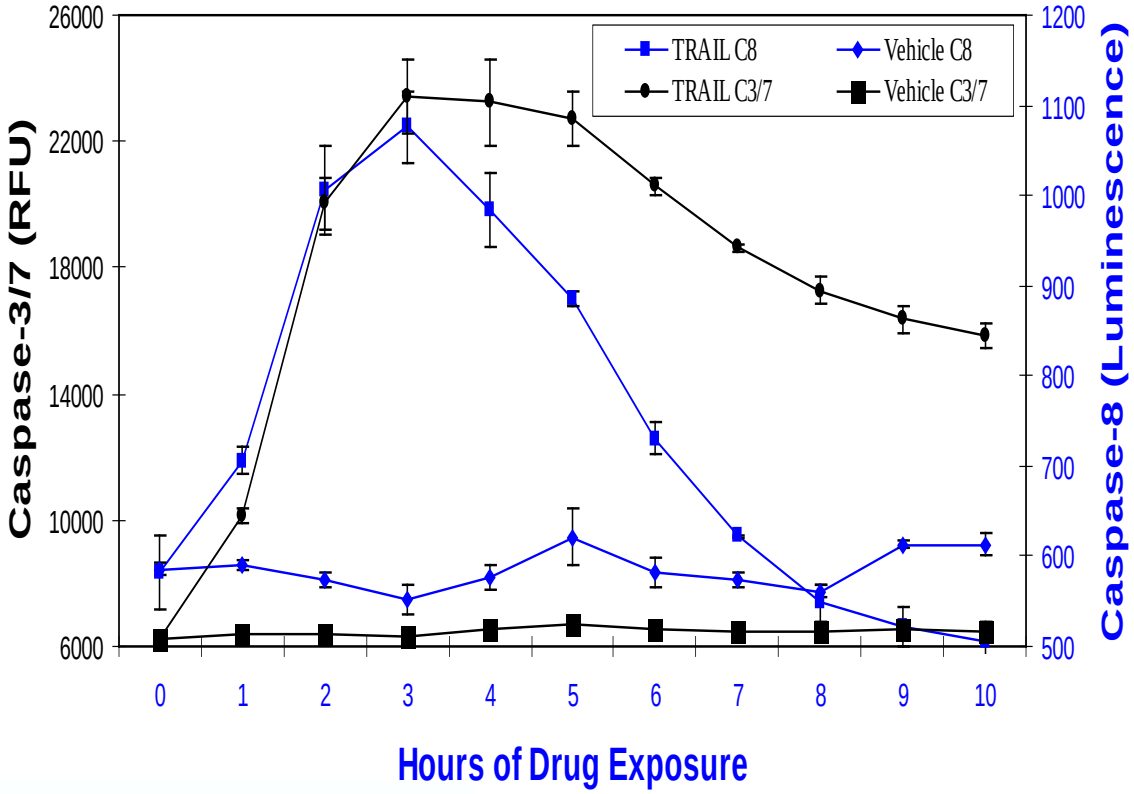


Caspase-Glo® 8 Reagent + $(Z\text{-}DEVD)_2$-R110

Incubate

Record Luminescence

Record Fluorescence



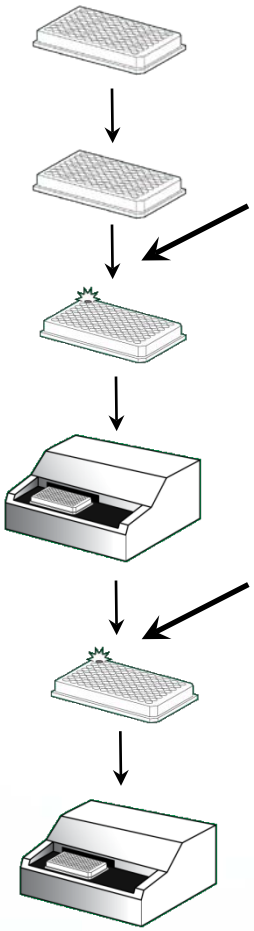
Mitochondrial toxicity can be detected by using controlled culture conditions

- Cells cultured in medium with galactose instead of glucose to eliminate production of ATP from glycolysis* (ATP production comes from mitochondrial oxidative phosphorylation)
- Expose cells to treatment less than 4hr
- Measure membrane integrity and ATP using sequential multiplex protocol
- Decrease in ATP without change in cell viability suggests mitochondrial toxicity

*Marroquin, L. D. et al (2007) Circumventing the Crabtree Effect: Replacing Media Glucose with Galactose Increases Susceptibility of HepG2 Cells to Mitochondrial Toxicants. *Toxicol. Sci.*97, 539-547.

Mitochondrial ToxGlo™ Assay
Multiplex membrane integrity and ATP content

Promega



Treat cells
30 min - 4hr
(glucose free)

bis-AAF-R110
Substrate

Incubate
30 min

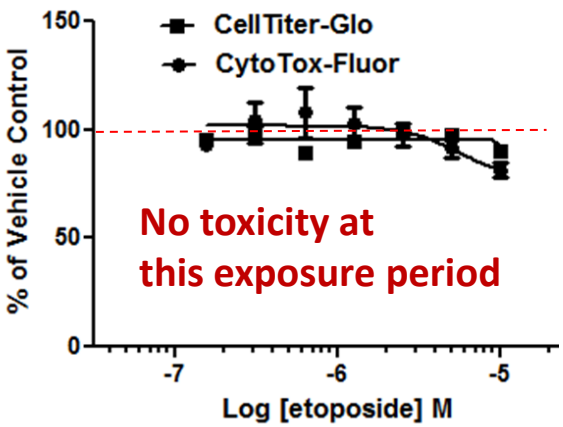
Record
Fluorescence

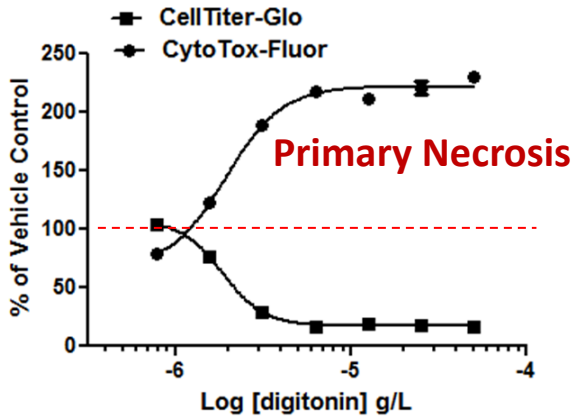
ATP Assay
Reagent

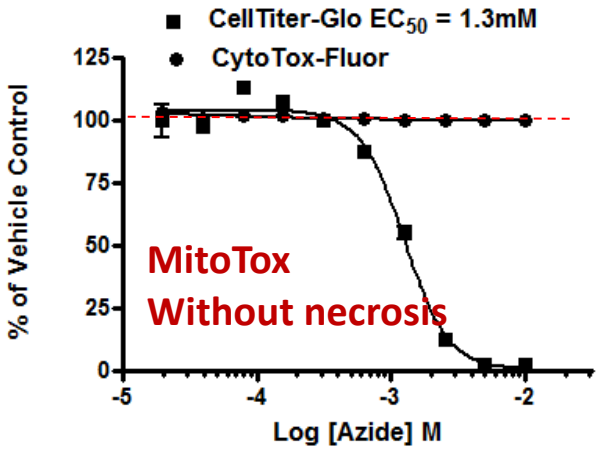
Incubate
10 min

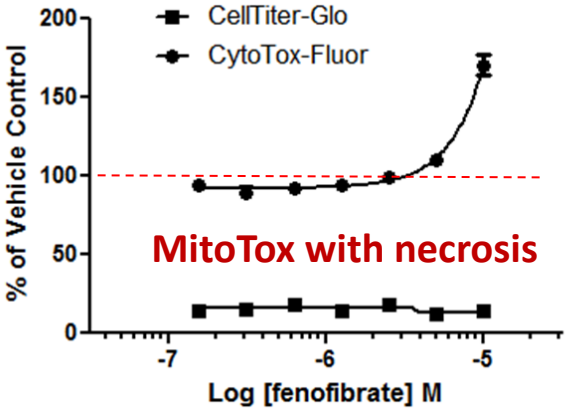
Record
Luminescence

Expected Assay Profiles





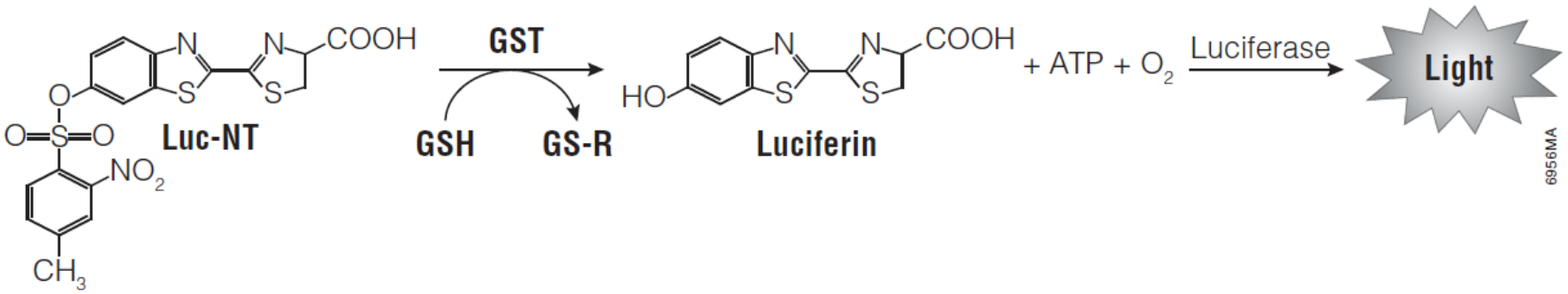




Oxidative stress marker assay

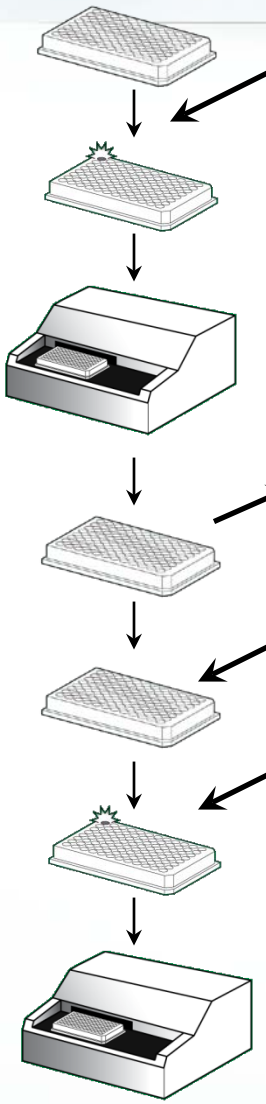
- Reduced glutathione (GSH) serves as an antioxidant in cells
- Low levels of GSH are associated with oxidative stress
- GSH can be measured with a luminescent assay using Glutathione S Transferase (GST) and luciferase
- Fluorescent cell viability assays can be sequentially multiplexed with the luminescent GSH assay

How the GSH-Glo™ Assay works



- Glutathione S Transferase transfers GSH to nitrotoluine group and converts pro-luciferin to luciferin in a GSH dependent reaction
- Luciferase reaction generates light proportional to the amount of GSH in cells

Multiplexing GSH levels with measuring viability



MultiTox-Fluor™ Reagent

Incubate 37°C

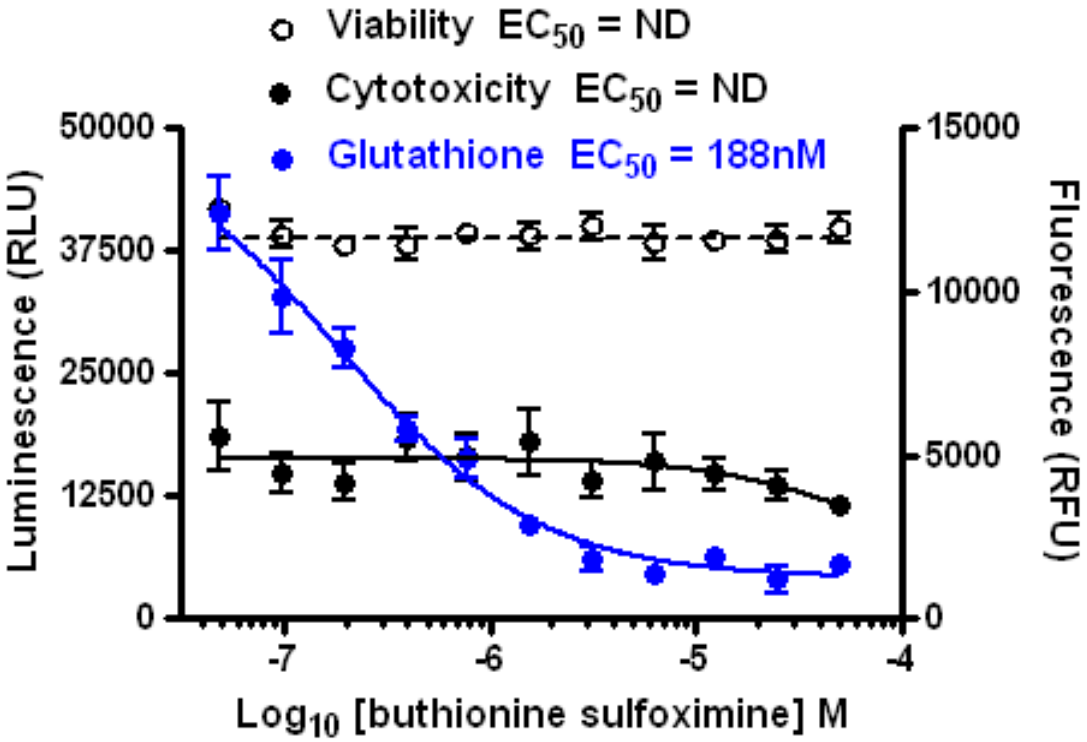
Record Fluorescence

Remove medium

GSH-Glo™ Buffer, 30min

GSH-Glo™ Reagents, 15min

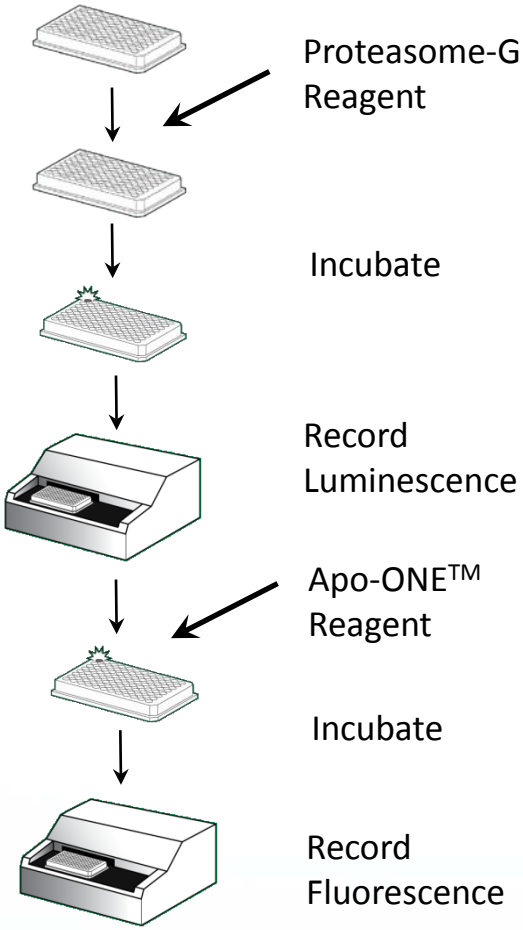
Record Luminescence

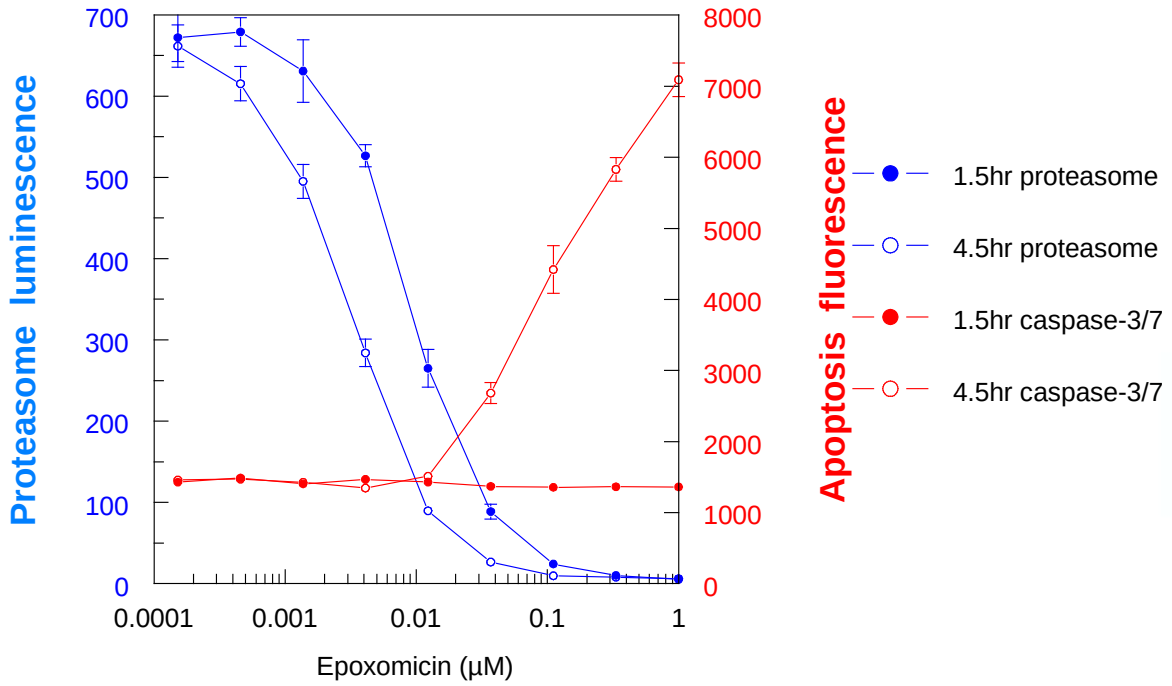


Proteasome activity as protein turnove marker

- The proteasome is responsible for most turnover of cytoplasmic proteins
- The proteasome is a therapeutic target for cancer
- Proteasome inhibitors (e.g. Velcade) selectively induce apoptosis in cancer cells
- Proteasome-Glo™ Assays provide adequate sensitivity to enable cell-based assays in HTS format
- Multiplexing proteasome and caspase assays can provide information on mechanism of toxicity leading to apoptosis

Multiplexing proteasome and apoptosis assays





Caspase-3 activity is absent after 1.5 hrs treatment with proteasome inhibitor; but increases after 4.5 hours incubation

Methods for measuring cytotoxicity

Promega

Overview of assays to measure viable cells

- MTT / MTS / Resazurin
- Protease marker
- ATP

Assays to detect dead cells

- LDH release
- Protease release
- DNA staining

Assays to measure apoptotic cells

- Caspase activity

Multiplex assays to measure early markers of cytotoxicity

- Viable, dead & apoptotic cells
- Extrinsic & intrinsic apoptosis pathways
- Mitochondrial toxicity
- Oxidative stress
- Proteasome activity

Luciferase reporters of cell stress pathways leading to cytotoxicity

Stress response pathways leading to cytotoxicity

- Stress response pathways are toxin activated signal transduction events that modulate transcription factors to trigger expression of cytoprotective genes to enable the cell to attempt to restore homeostasis.*
- Triggering cell response pathways occurs at lower toxin doses or exposure times than what is needed to directly trigger necrosis or apoptosis.
- If stress cannot be overcome to re-establish homeostasis, the result is induction of apoptosis and removal of the cell.

*Simmons, S.O. et al., Cellular stress response pathway system as a sentinel ensemble in toxicological screening. Tox. Sci. 111(2): 202-225, 2009.

Luciferase reporters of cell stress pathways leading to cytotoxicity

Promega

- Luciferase reporter assays are ideal to measure effects of compounds on expression of stress pathway genes.
- Reporter assays can help identify the cellular mechanisms used to adapt to a perturbation, gaining insight into the mechanism of toxicity caused by the compounds.
- Promega has developed luminescent reporter gene assays directed at the major pathways involved in cellular stress.
- Several vectors and stable cell lines are available from Promega as “Latest Research Materials”

Stress and toxicity pathway vectors

Promega

Pathway/Response	Transcription Factor	Name
Antioxidant	Nrf2	pGL4/ARE
DNA damage	p53	pGL4/p53
ER stress	ATF6	pGL4/ERSE
ER Stress	ATF4	pGL4/ATF4
ER stress	Xbp1	pGL4/Xbp1
Heavy metal stress	MTF1	pGL4/MRE
Heat shock	HSF1	pGL4/HSE
Hypoxia	Hif1α	pGL4/HRE
p38/JNK	AP1	pGL4/AP1
Xenobiotic stress	AhR	pGL4/XRE
Inflamation	NFκB	Cat # E8491
Osmotic stress	NFAT5	pGL4/NFAT5

All constructs with pGL4.27 backbone [luc2P/minP/HygR]

Examples of major stress response pathways

Oxidative Stress Response: Signaling pathway that leads to Nrf2 transcription factor binding to antioxidant response elements (ARE) that induce expression of genes to neutralize Reactive Oxygen Species (ROS) and to limit oxidative damage to cellular components.

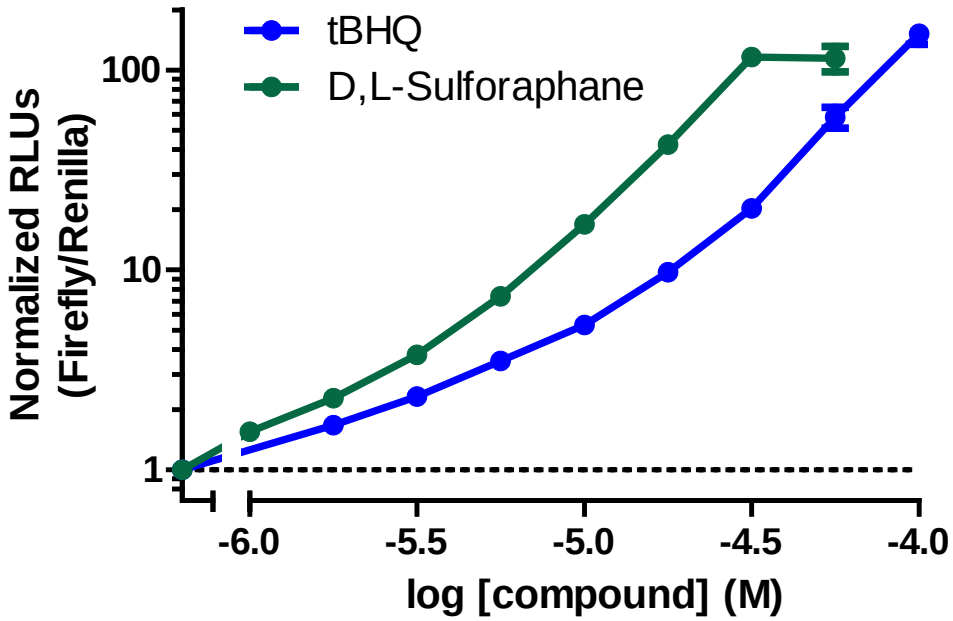
Heat Shock Response: HSF-1 activates expression of Hsp70 & Hsp27 chaperones that bind to and facilitate refolding of denatured proteins.

Examples of major stress response pathways (cont.)

p53-mediated DNA Damage Response: A genotoxic response pathway mediated by the p53 transcription factor to arrest the cell cycle, regulate DNA repair, and if the damage is too great, mediate apoptosis.

ER Stress Response: ATF6 directs transcription of endoplasmic reticulum (ER) specific chaperones and slowing of general protein synthesis to enable ER to refold unfolded proteins.

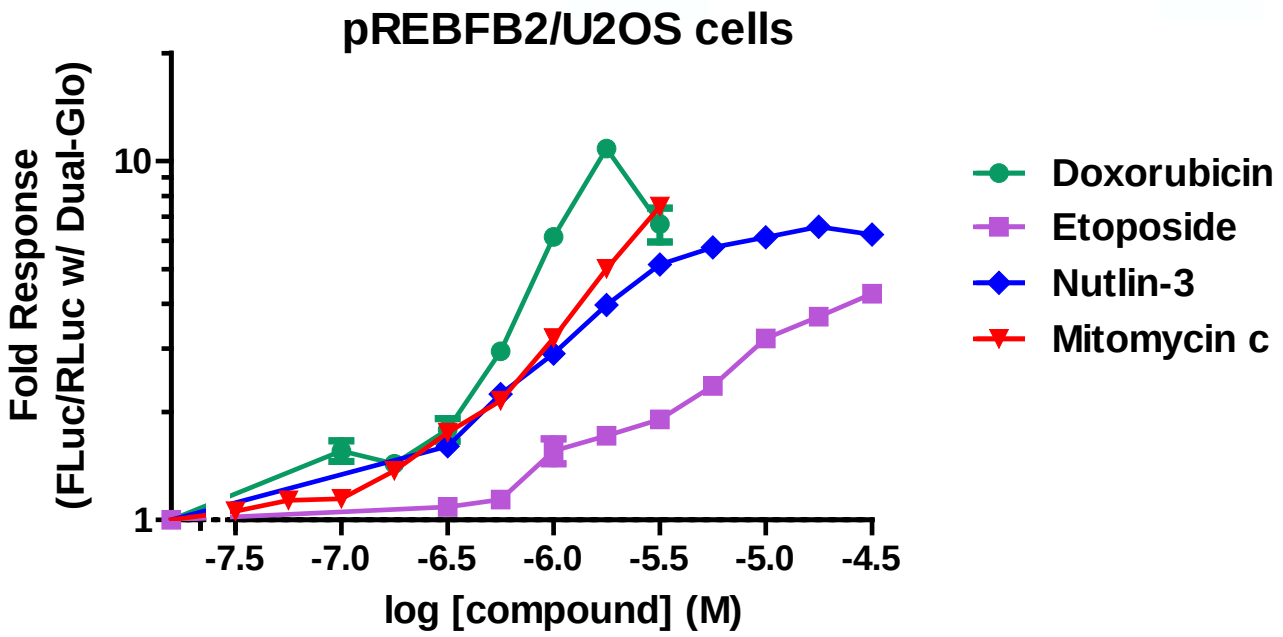
Oxidative stress response / ARE-luc2P (Nrf2)



ARE	D,L-sulf	tBHQ
S/B	116	152
EC50 (uM)	19.6	NA

Data collected 18 hrs post compound addition (pREBFB25/HEK293)

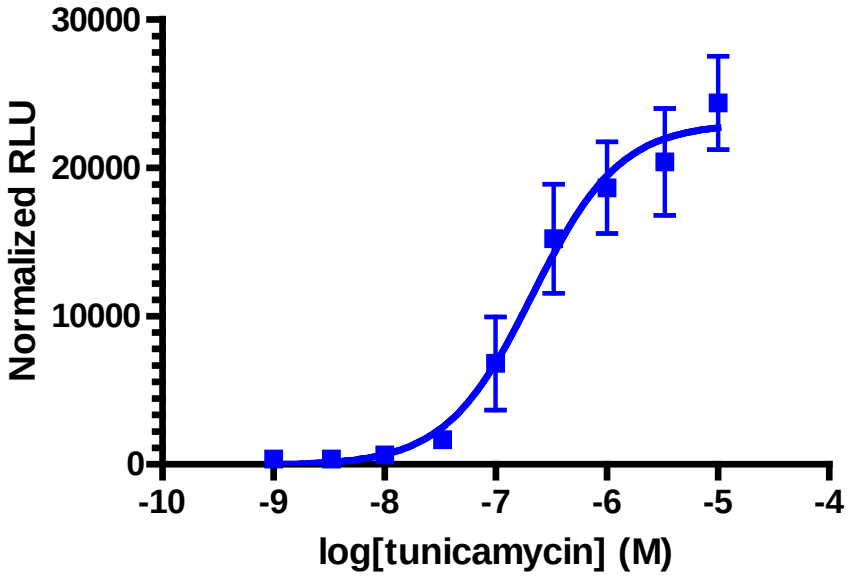
DNA damage stress response / p53-luc2P (p53)



	Doxorubicin	Etoposide	Nutlin-3	Mitomycin c
S/B	10.9	4.3	6.6	7.5
EC50 (uM)	0.8	13	1.5	NA

Data collection 18 hrs post-treatment for all except mitomycin c (40 hrs)

ER stress response / ERSE-luc2P (ATF6)



	RE28
S/B	71
EC50 (nM)	216

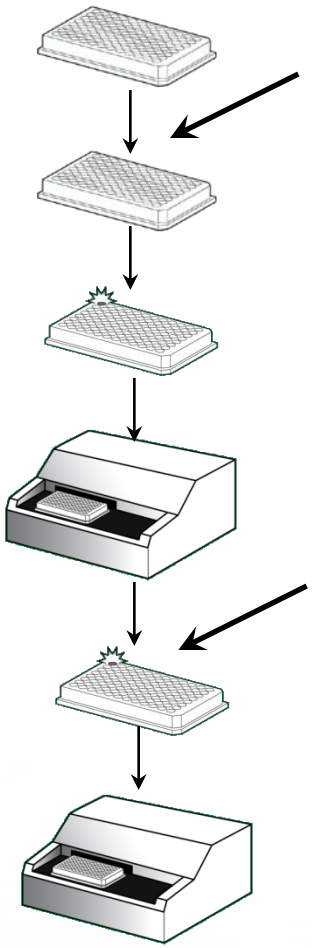
Data collected 23 hrs post addition of tunicamycin (pREBFB28/HeLa)

Several stress response reporters can be tested in parallel

Protocol to profile a compound against RE panel

- Add each DNA to FuGENE® HD in Opti-MEM I®
- Add cells to transfection complexes
- Dispense cells into 96-well plate, with each RE in its own column
- Incubate overnight
- Treat cells 18 hr with varying doses of the compound of interest
- Multiplex assays for cell viability and reporter induction

Multiplex cell viability & cell stress reporter genes



CellTiter-Fluor™ Reagent (GF-AFC)

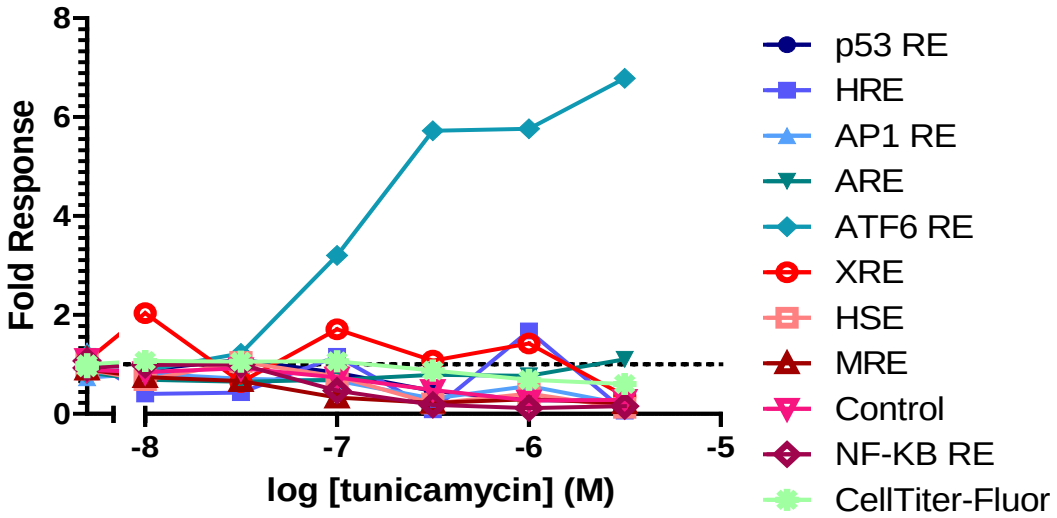
Incubate 37°C 15-30 min

Record Fluorescence

ONE-Glo™ Reagent

Record Luminescence

HepG2 cells treated 18 hrs
Tunicamycin selectively stimulates
ATF6 Response Element



Excellent multiplexing partners for live cells or dead cells

Promega

CellTiter-Fluor™ Assay

- Reaction occurs within living cells.
- Live cell protease is inactive outside cells.
- AFC fluorescence is proportional to live cells


ScienceDirect

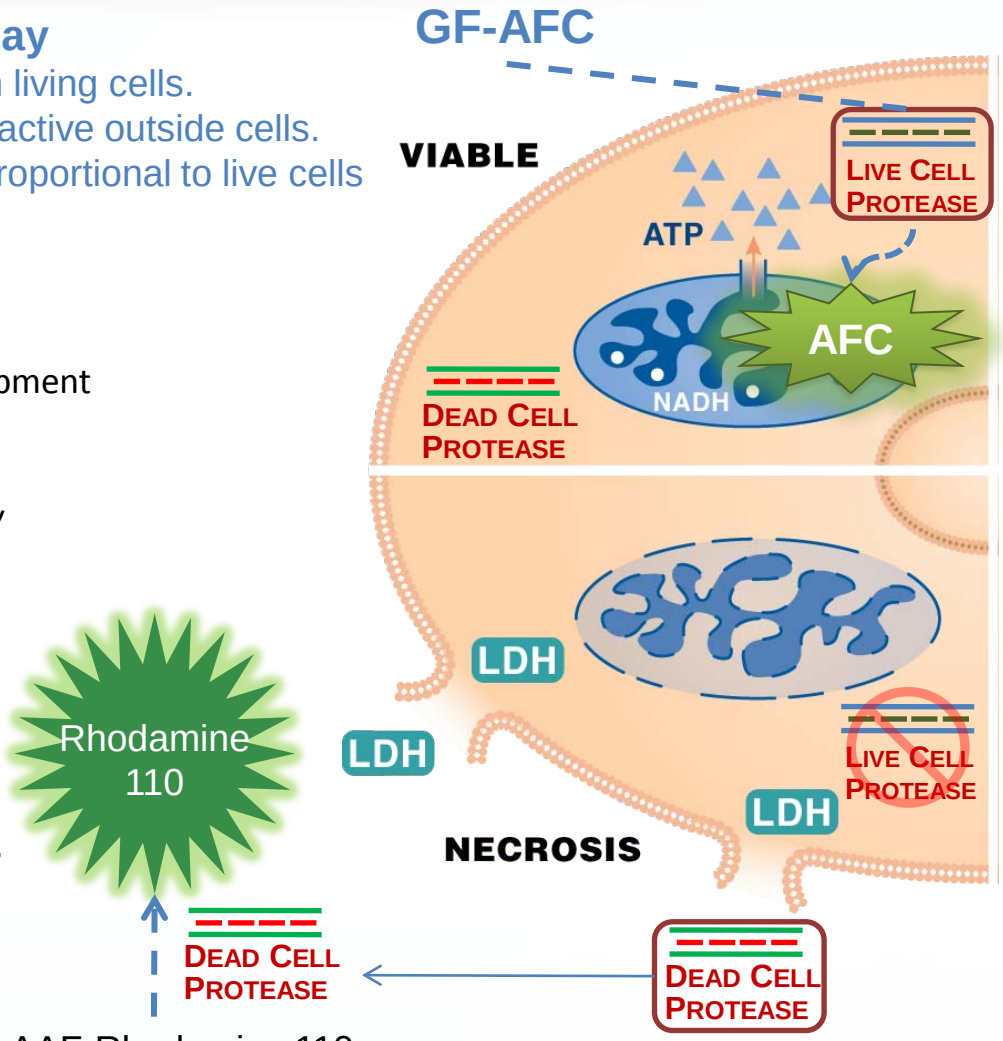
A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers

Andrew L. Niles, Richard A. Moravec, P. Eric Hesselberth, Michael A. Scurria, William J. Daily, Terry L. Riss

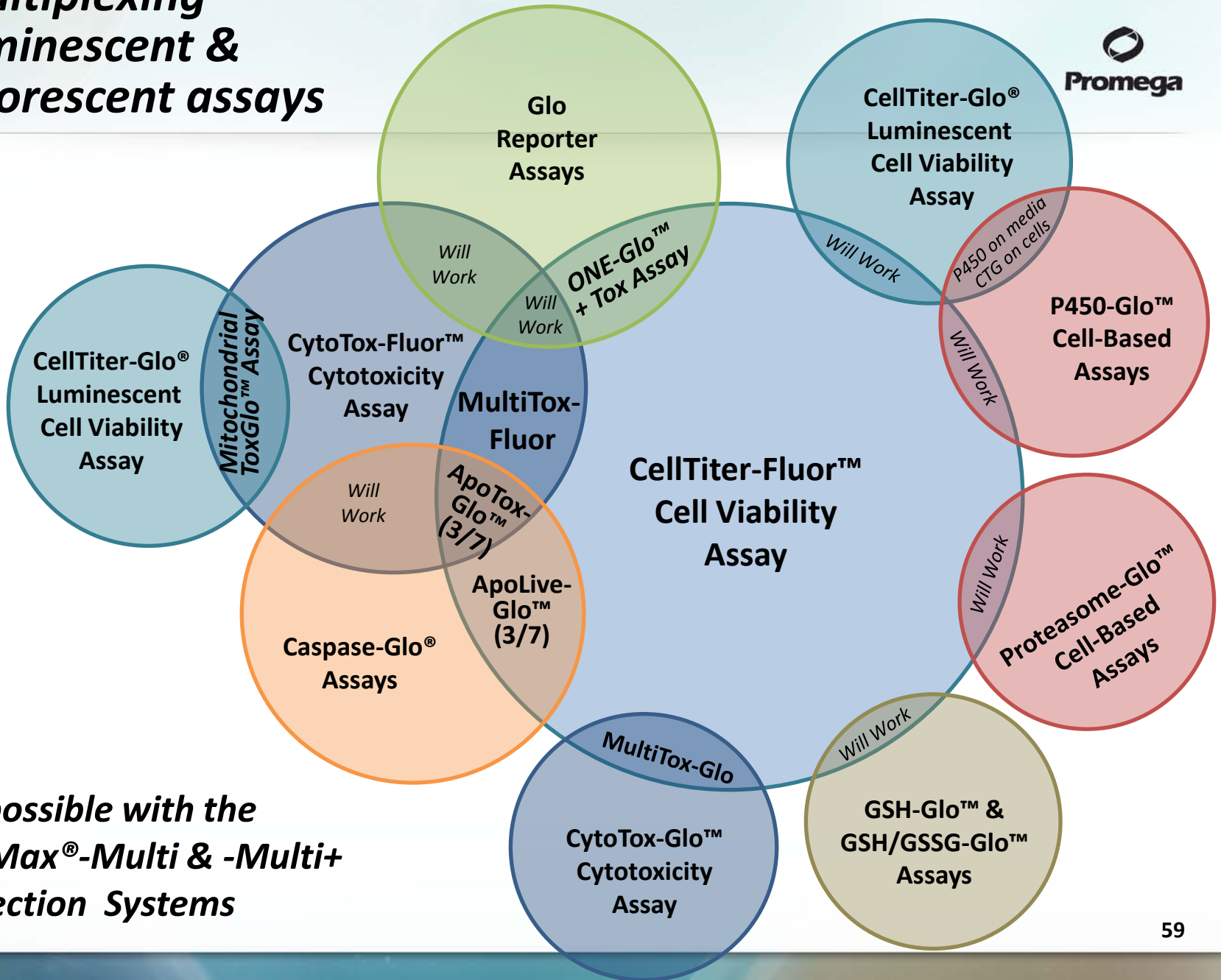
Read about the development of these assays:
Niles, A.L., *et al.* (2007)
Analytical Biochemistry
366, 197-206.

CytoTox-Fluor™ Assay

- Rhodamine 110 substrate cannot enter cells.
- Reaction occurs in the culture medium.
- Rhodamine110 fluorescence is proportional to dead cells.



Multiplexing luminescent & fluorescent assays



All possible with the GloMax®-Multi & -Multi+ Detection Systems

Summary

- There is a variety of multi-well plate assay options available to detect more than just whether cells are alive or dead
- Biochemical and cell-based assays are available to detect cell stress events (e.g. oxidative stress, mitochondrial toxicity, proteasome, etc.)
- Luciferase reporter gene assays and stable cell lines have been developed to study effects on the major stress pathways leading to cytotoxicity
- Multiplex detection of cell viability in combination with luciferase reporters or other biochemical marker assays provides powerful tools to study the mechanisms leading up to cytotoxicity

Product names for assays described today

Assay Technology	Promega Product Name
MTT	CellTiter 96® Non-Radioactive Cell Proliferation Assay
MTS	CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay
Resazurin	CellTiter-Blue® Cell Viability Assay
Protease marker	CellTiter-Fluor™ Cell Viability Assay
ATP	CellTiter-Glo® Luminescent Cell Viability Assay
LDH	CytoTox-ONE™ Homogeneous Membrane Integrity Assay
Protease release	CytoTox-Fluor™ Cytotoxicity Assay
DNA staining	CellTox-Green (Inquire; under development)
Caspase-3/7 activity	Caspase-Glo® 3/7 Assay
Caspase-8 activity	Caspase-Glo® 8 Assay
Caspase-9 activity	Caspase-Glo® 9 Assay
Mitochondrial toxicity	Mitochondrial ToxGlo™ Assay
Oxidative stress	GSH/GSSG-Glo™ Assay
Proteasome activity	Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay
Live+Dead+Apoptotic	ApoTox-Glo™ Triplex Assay
Stress response pathways	Luciferase reporters of cell stress pathways (Inquire in Latest Research Materials section of promega.com)

Questions Welcome

Promega